Enterococcus infection of Caenorhabditis elegans as a model of innate immunity

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Abstract

The vast majority of metazoan species are invertebrates, which lack an adaptive immune system for defense against microbes. Instead, their innate immune system is sufficient for defense against pathogenic organisms, which are recognized through the perception of microbe-associated molecular patterns (MAMPs), such as peptidoglycan and flagellin. MAMPs, however, are common to all microbes, pathogenic as well as commensal, and it is becoming clear that the host differentiates between the two by sensing the impact of the infection on host cellular physiology, highlighting the crosstalk between sensing of infectious microbes and host damage in the immune system. In this thesis, I investigated these interactions using the *Caenorhabditis elegans* infection model with two related enterococcal species, *Enterococcus faecalis*, which causes a lethal infection in *C. elegans*, and *Enterococcus faecium*, which does not increase host mortality. Ultrastructural imaging revealed that during infection with either *E. faecalis* or *E. faecium*, the intestine becomes distended with proliferating bacteria, and that this occurs in the absence of obvious host cellular damage. Using genetics, whole-genome transcriptional profiling, and multiplexed gene expression analysis, I demonstrated that *C. elegans* mounts a transcriptionally-driven defense response to both live and heat-killed enterococcal species that is potentially triggered by MAMPs, and that pre-exposure to heat-killed *Enterococcus* protects *C. elegans* against a subsequent *E. faecalis* infection. I also found that the *C. elegans* host defense response to *E. faecium* shows a greater dependence upon stress response signaling pathways than the *C. elegans* host response to *E. faecalis*. These results provide evidence for the ability of *C. elegans* to respond to MAMPs in a manner dependent upon previously identified immune signaling pathways. Additionally, these studies show that *E. faecium* is a weak *C. elegans* pathogen, and that an active host defense response is required to keep *E. faecium* at bay. The data presented provide evidence for the underlying differences in the host response and, potentially, virulence mechanisms,

between *E. faecalis* and *E. faecium* infection of *C. elegans*. The paradigm of extensive crosstalk between conserved pathways that sense MAMPs, xenobiotics, and host stress observed in this thesis is likely to apply to metazoans in general.

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To my parents.

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If you know the enemy and know yourself,
you need not fear the result of a hundred battles.
If you know yourself but not the enemy,
for every victory gained you will also suffer a defeat.
If you know neither the enemy nor yourself,
you will succumb in every battle.

Sun Tzu

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Innate immunity in *Caenorhabditis elegans*

Even the ever-so-simple worm has an immune system, just not the cellular immune system of larger metazoans that can sacrifice some of its foot soldiers in the fight against pathogens. Since there are no circulating cells in *Caenorhabditis elegans*, each cell has to look out for itself. The adult *C. elegans* is comprised of a finite number of post-mitotic sessile cells, many of which are specialized to perform very specific functions.

Innate immunity in vertebrates is thought to be mediated by pattern recognition receptors (PRRs) that recognize invariant molecular structures shared by pathogens, called microbe-associated molecular patterns (MAMPs), such as flagellin, lipopolysaccharide, and unmethylated CpG motifs[92]. In

vertebrates, Toll-like receptors (TLRs) are the predominant transmembrane PRR family. Upon the recognition of MAMPs, TLRs activate a downstream MAPK signaling cascade, which causes the nuclear translocation of the NF-κB and IRF transcription factors, culminating in the expression of inflammatory cytokines and type I interferons, and ultimately the production of immune effectors that inhibit the infection. Recent studies have also demonstrated the importance of cytosolic PRRs, namely the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD) proteins, and nucleotide-binding site leucine-rich repeat-containg proteins (NLRs), which detect MAMPs in the cytosol[91].

MAMP recognition alone, however, does not explain how the innate immune system can distinguish between pathogenic and non-pathogenic microbes, since MAMPs are present on non-pathogenic as well as pathogenic bacteria. It has been known that during an infection, pathogens can actively perturb the properties and behavior of a cell, for instance by modifying plasma membrane permeability, or inducing apoptosis, which activates the immune system via endogenous or exogenous signals, called damage-associated molecular patterns (DAMPs)[224]. Furthermore, the localized context of infection is also important for innate immune recognition of a pathogen. One example is the exclusive expression of TLR5 on the basolateral surface of intestinal epithelia so that only flagellin that contacts the basolateral epithelial surface will generate a proinflammatory response[50]. This allows for the immune system to recognize pathogenic *Salmonella*, but not commensal *E. coli*, as the former translocates flagellin across epithelia. Additionally, while MAMPs are often thought to be invariant bacterial features, pathogens are often able to vary their MAMPs; for instance, many variants of lipopolysaccharide exist across different bacteria[176].

It has been proposed that the immune system may respond to MAMPs in the context of other signals, derived from either the host or the pathogen, that are only generated during a pathogenic infection. These ideas have been incorporated into a revised model of pathogen detection, whereby MAMPs are recognized in the context of additional signals that derive from common "patterns of pathogenesis" employed by pathogens to infect, multiply within, and spread among their hosts[224].

Complementary recognition of such patterns of pathogenesis, in concert with MAMP recognition, would allow the host to distinguish virulent from avirulent organisms, and may thus allow the host immune system to respond commensurately to the virulence level of the pathogen. In support of this model, there is increasing evidence for the existence of host signaling pathways that can respond to bacterial secretion systems and virulence-associated effectors, including toxins.

1.1 Rationale for studying host-pathogen interactions in invertebrate models

Understanding the relationship between microbial virulence mechanisms and the host response has helped to form an integrated view of microbial pathogenesis. Given that multiple interconnected factors with complex inter-relationships between both the host and the microbe are at play, the combined use of genetically tractable model hosts and pathogens resulting in a dual genetic model makes it easier to deconstruct host-pathogen interactions with greater facility and in greater detail than is possible using vertebrate models[38].

Invertebrate infection models have revealed important insights into host-pathogen interactions, including guiding principles underlying both the infectious process and the host immune response that are relevant to mammalian infections[13,104,230]. The value of using simple invertebrate hosts to study innate immunity and host-pathogen interactions is enhanced by the evolutionary conservation of both ancient innate host defenses and bacterial virulence mechanisms. In general, the shared use of virulence factors by a pathogen infecting different hosts suggests that the mechanisms of pathogenesis are often host-independent[233,79]. Similarly, if a particular pathogen activates similar defense responses in both invertebrate and vertebrate hosts via conserved signaling pathways, it suggests that the underlying mechanisms of innate immunity may be conserved across metazoans. Thus, while the specific immune responses activated in a model host are likely to be most representative of phylogenetically related species, the categories of responses and the signaling pathways involved may be more highly conserved and may reflect common features of metazoan infection biology (Figure 1.1)[170].

Figure 1.1: Man is but a worm. A cartoon by Linely Sambourne, published in the satirical magazine Punch, published on December 6, 1881, short after the publication of Charles Darwin's *On the Origin of Species*. In the cartoon, the evolution of a segmented worm into a human – here, Darwin himself – to ridicule the link between the two disparate species.

1.1.1 *C. elegans* as a model host

The simplicity of the small, bacteriovorous roundworm *C. elegans* has allowed it to become an attractive system for the study of innate immunity. By replacing non-pathogenic *E. coli*, the normal laboratory food source for *C. elegans*, with a pathogenic microbe, one can activate the worm innate immune system. Its genetic tractability easily allows for thorough analysis of genetic pathways, especially using whole-genome RNAi screens[89,88].

The small, free-living nematode *C. elegans* lies at a unique point in evolution in terms of its immune system. About 600 million years ago, *C. elegans*, along with other bilaterians, diverged from cnidarians and choanoflagellates at the junction of a common eumatazoan ancestor, and 30 million years later, the bilaterally-symmetric invertebrates (which include the fruitfly *Drosophila melanogaster* and *C. elegans*) diverged from the vertebrate lineage (including mammals). By comparing a number of signaling proteins in innate immunity, it was discovered that while the genomes of humans, mice and *Drosophila* contained homologs of the immune adaptor MyD88 and the master transcriptional regulator NF-κB, the *C. elegans* genome did not. Given that MyD88 and NF-κB were present in the genomes of choanoflagellates and cnidarians, the most straightforward explanation is that an ancestor of *C. elegans* had lost these molecules in evolution[79]. If *C. elegans* did not have these key immune signaling molecules in their genomes (and additionally has no motile cells, or even a circulatory system), how exactly does *C. elegans* defend itself in the wake of pathogenic attack?

One might propose that perhaps *C. elegans* has survived for hundreds of millions of years without an immune system. This is extremely unlikely, as every organism in the world, down to the simple prokaryote, is susceptible to infection. While bacteria are at risk to infection by bacteriophage, even some viruses are susceptible to "infection" by other viruses[100]. *C. elegans* has many potential pathogens in its environment: in the wild, *C. elegans* inhabits decaying vegetation and fruit and thus encounters a diverse microcommunity, rich in microbes[43]. As its environment naturally contains noxious bacteria, *C. elegans* presumably recognizes the presence of the pathogenic bacteria in its food

and environment in order to mount effective immune and detoxification responses.

1.2 Immune signaling in *C. elegans*

Immune signaling in *C. elegans* has been studied in the context of the intestine and epidermis in a number of model pathosystems using diverse pathogens. The molecular players that control the multifaceted *C. elegans* immune response were initially characterized using a forward-genetics approach to screen for mutants that were hypersensitive to pathogenic infection. Later, whole-genome, reverse-genetic screens using feeding RNAi and proteomics were used to dissect the upstream and downstream components of these immune pathways in *C. elegans*.

1.2.1 Immunity in the *C. elegans* intestine

Following ingestion by *C. elegans*, bacteria travel through the alimentary system of the worm, which includes the intestine, the main site of immune function against ingested pathogens. The adult *C. elegans* worm has 20 non-renewing intestinal epithelial cells (IECs) that share many common morphological features with mammalian IECs, including a "brush border" of microvilli, anchored into a cellular structure called a terminal web[126]. As the worm has no motile immune cells, it is dependent on these IECs to mediate intestinal immunity, and it is these cells that secrete antimicrobial peptides, C-type lectins, lysozymes and ShK-like toxin to kill pathogenic bacteria[79].

C. elegans responds to pathogens through at least six parallel immune signaling pathways, a number of which are canonical signaling pathways conserved in metazoans: (i) a conserved PMK-1 p38 MAPK pathway, which confers broad-spectrum resistance to many pathogens[96], (ii) a DAF-2/DAF-16 insulin-like growth factor pathway, which confers broad-spectrum resistance when constitutively activated[48,15], (iii) a *Serratia marcescens*-activated pathway overlapping with the *C. elegans* DBL-1 TGF-β pathway[122], (iv) a *S. aureus*-activated pathway using components of the Wnt signaling pathway, including the *C. elegans* β-catenin homolog BAR-1 and the downstream homeobox transcription factor EGL-5[77], (v) a *P. aeruginosa*-activated pathway comprising the bZIP transcription factor

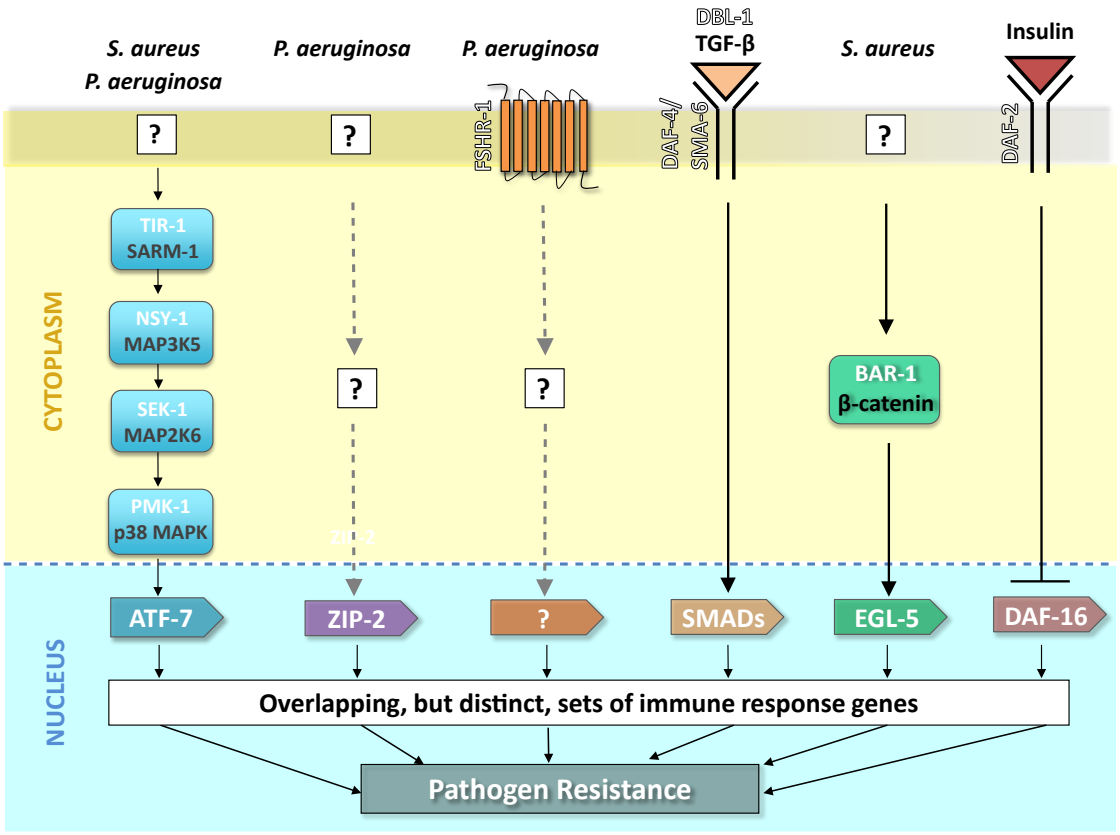


Figure 1.2: Immune signaling pathways in *C. elegans*. Multiple signaling pathways mediate intestinal immunity in *C. elegans*. The nematode coordinates pathogen-specific defense responses against ingested bacteria through a number of parallel pathways.

ZIP-2[31,36], and (vi) a pathway containing the G-protein coupled receptor FSHR-1, homologous to the mammalian follicle-stimulating hormone receptor[163] (Figure 1.2).

Mitogen-activated protein kinase (MAPK) pathways

The most well-characterized of these *C. elegans* immune pathways is the PMK-1 p38 MAPK cascade, which was identified in a genetic screen for mutants that displayed enhanced susceptibility to *P. aeruginosa*[96]. The protein members in this cascade are NSY-1, SEK-1, and PMK-1, which are homologous to the ASK1 MAPKKK, MKK3/6 MAPKK and p38 MAPK, respectively, in mammals. One of the functions downstream of *C. elegans* p38 MAPK signaling is the activation of ATF-7, a transcriptional regulator orthologous to ATF2/ATF7 in mammals, which regulates the induction of a number of immune effectors important for defense against *P. aeruginosa*[194]. Additionally, PMK-1 is also involved in the activation of the Nrf family transcription factor SKN-1 to mediate oxidative stress resistance[76]. Oxidative stress is a particularly dangerous cellular stress, as high quantities of ROS can disrupt DNA, protein, and lipid integrity. ROS is produced endogenously by the cell's mitochondria, usually due to inefficiencies in oxidative phosphorylation, as well as during ER stress, as the enzymes that form disulfide bonds via the oxidation of cysteine residues also generate hydrogen peroxide. Moreover, some bacteria have also been known to produce ROS, including *Enterococcus*[72,232,203]. Given these findings that PMK-1 is involved in both immune and stress response pathways, it possible that there is some crosstalk between these pathways, or at least pleiotropy in the role of PMK-1. Regarding this former possibility, it has been demonstrated that the activation of PMK-1 by phosphorylation can also be induced by the MAPK kinase MEK-1, which has been described to be activated by heavy metal stress in *C. elegans*[97].

Several proteins, TIR-1 (a homolog of the human SARM protein), EGL-30 ($G_{q\alpha}$), EGL-8 (PLCβ), TPA-1 (PKCδ) and DKF-2 (protein kinase D), have been found to act upstream of the NSY-1/SEK-1/PMK-1 cascade, but the literature becomes a bit murky from this point[93,244]. The involvement of the $G_{q\alpha}$ protein EGL-30 implies the role of a G-protein coupled receptor and its ligand in intestinal

immunity, but where does TIR-1 act in relation to EGL-30 – in the same or a parallel pathway? If it acts upstream of EGL-30 or in a parallel pathway, what molecules are upstream of TIR-1? TIR-1 is named for the homotypic TIR domain that it contains, indicating that it likely interacts with another TIR-domain containing protein. However, the only other TIR-domain containing protein in *C. elegans* is TOL-1, the sole Toll receptor in *C. elegans*, which, strangely enough, does not appear to have an immune role. In previous studies, two alleles of *tol-1* have been analyzed. The first allele has a deletion in part of the extracellular domain and has a severe developmental defect. The second allele has a deletion in the intracellular TIR signaling domain and is predicted to be a hypomorph because it lacked an ostensible developmental defect. While this second allele is considered to be null for conventional immune signaling[166], it exhibits no defect in the induction of host response genes to various bacterial and fungal pathogens[247]; a decrease in survival for this second *tol-1* mutant was observed on *Salmonella enterica*[212], in addition to decreased production of antimicrobial peptides, though it has been argued that these defects may still be of a developmental origin.

A second MAPK cascade shown to have a role in *C. elegans* immunity is the extracellular signal-regulated kinase (ERK) pathway, which is involved in the resistance of *C. elegans* to the Gram-positive bacterium *Microbacterium nematophilum*, a nematode-specific bacterial pathogen that adheres to the posterior alimentary tract of the worm[148]. In response to *M. nematophilum* infection, *C. elegans* activates MPK-1, a ERK1/2 MAPK homolog, to mount a protective swelling response in the posterior intestinal cells and anus of the worm.

Insulin-like receptor pathway

The insulin-like receptor DAF-2 and its associated Foxo family transcription factor DAF-16 are important for *C. elegans* lifespan and stress tolerance, but their exact roles in immunity are less well understood. When active, DAF-2 holds DAF-16 inactive in the cytoplasm, and in the absence of DAF-2, DAF-16 is able to translocate into the nucleus. Inactivation of *daf-2* causes worms to not only be long-lived, but also to have enhanced resistance to several bacteria, including *P. aeruginosa* and

E. faecalis[48]. Interestingly, while loss-of-function mutations in the serine/threonine kinases (PDK-1, SGK-1, AKT-1, and AKT-2) that lie downstream of the DAF-2 receptor dramatically lengthens the lifespan of these worms, inactivation of only *akt-1* and *akt-2* renders worms more resistant to *P. aeruginosa*[37], providing one example that longevity and pathogen resistance can be decoupled. While it has been thought that *daf-2* mutants are resistant to pathogens because they overexpress a number of antimicrobial genes, in actuality, most of the genes upregulated during infection with *P. aeruginosa* are not DAF-16-dependent, but PMK-1-dependent[192,216]. Moreover, genes downstream of PMK-1, which have been thought to have an immune function, appear to be repressed by DAF-16, and DAF-16 does not translocate into the nucleus after *P. aeruginosa* infection (in contrast to what has been observed of DAF-16 following abiotic cellular stresses). DAF-16, however, is required for the enhanced pathogen resistance of the *daf-2* mutant[48], an observation that strongly supports the idea that DAF-16 still promotes certain immune responses – just not the ones that are regulated by PMK-1. Another explanation is that DAF-16's role in pathogen resistance really stems from its role in a general stress response. As insulin signaling pathway regulates many different facets of *C. elegans* physiology, it may be difficult to tease out the exact role of DAF-2/DAF-16 in *C. elegans* immunity.

TGF-β signaling

In the response to bacterial infections, *C. elegans* also activates the TGF-β homolog DBL-1 (decapentaplegic/bone morphogenic protein line-1) to mediate a protective response against pathogenic attack[122]. In addition to DBL-1, the other members of the *C. elegans* TGF-β pathway include its two membrane anchored receptors SMA-6 and DAF-4, as well as three cytoplasmic signal transducers SMA-2, SMA-3 and SMA-4[39]. Previous studies noted a common overlap in targets between the DBL-1 pathway and the genes upregulataed by infection with the Gram-negative pathogen *Serratia marcescens*, suggesting that *S. marcescens* may activate these gene targets in a DBL-1-dependent manner[122].

Other immune signaling pathways

A number of other immune signaling pathways have been identified in *C. elegans*, though many of the upstream activators of these genes remain to be identified. The *C. elegans* β-catenin BAR-1 and the homeobox transcription factor EGL-5 are critical for defense against *S. aureus*[77]. Also required for *C. elegans* defense against *S. aureus* is the transcription factor HLH-30 (homologous to TFEB in mammals), which is required for the expression of nearly 80% of *S. aureus*-activated genes in *C. elegans*[229]. The bZIP transcription factor ZIP-2 is necessary for the induction of a number of *P. aeruginosa*-induced genes[31,36]; additionally, translational inhibition by *P. aeruginosa* ribotoxin ToxA is sufficient to activate ZIP-2, a point I will return to later in this chapter. The leucine-rich repeat-containing G-protein coupled receptor FSHR-1 (homologous to follicle stimulating hormone receptor in mammals) has also been demonstrated to control a set of *P. aeruginosa*-induced genes through its activity in the intestine[163].

1.2.2 Immunity in the *C. elegans* epidermis

The epidermis, also called the hypodermis in *C. elegans*, serves as a physical barrier against mechanical damage and pathogenic infection. In *C. elegans*, the internal tissues and organs are sheathed by a single-celled epithelial barrier formed from multinucleate syncytia (cell fusions) during development[17]. In the epidermis, transepidermal hemidesmosones act to bridge the apical side (the cuticle, which forms the resilient, collagenous exoskeleton) to the extracellular matrix of the basal side, resulting in the cuticle, epidermis, and muscles being fastened through intermediate filaments. *C. elegans* epidermal immunity has been extremely well characterized, demonstrating the power of genetics in probing innate immune pathways. The pathogens described in the previous section enter the worm through ingestion and thus interface with the intestinal lumen, which is already equipped in some respect for killing microbes, as *C. elegans* is naturally bacteriovorous and digests microbes to gain nutrition.

There are, however, pathogens that infect *C. elegans* via its cuticle, the best characterized being *Drechmeria coniospora*, a natural fungal pathogen of nematodes. To infect the worm, *Drechmeria* first pierces through its cuticle, allowing hyphae to grow and penetrate through the *C. elegans* epidermis[82,161], which initiates a complex transcriptional program in reaction to both the fungus itself and to the wound, two responses that appear to be regulated independently[165]. The pathway activated by fungal infection requires the Tribbles-like kinase NIPI-3, but its most upstream receptor in this pathway remains unknown[244]. However, the upstream receptor of the pathway activated by cuticle wounding has recently been identified as a nematode-specific G-protein coupled receptor called DCAR-1 (dihydrocaffeic acid receptor-1, a homolog of the human cholecystokinin A receptor) that binds the endogenous ligand HPLA (4-hydroxyphenyllactic acid), a tyrosine derivative[246]. The receptor and its ligand were shown to be specifically required for the immune and wounding response to *Drechmeria* in a tour-de-force study that implicated these two molecules in damage recognition. As the GPCR family is much larger in *C. elegans* than in any other organism with 1,300 members, this study, which showed that a GPCR plays a key role in pathogen recognition, suggested that other GPCRs may also be involved in the immune response. In the end, the activation of NIPI-3 in the fungal infection-specific pathway, and the activation of DCAR-1 (GPCR) through its interaction with RACK-1 (G_β), GPA-12 (G_α), and EGL-8 (phospholipase C), converge upon TPA-1 (PKCδ) to ultimately activate TIR-1 and the p38 MAPK cascade to induce fungal-specific antimicrobial peptides in the *C. elegans* epidermis[33].

From studies initially investigating *Drechmeria* infection of *C. elegans*, it was shown that sterile damage (in the absence of a pathogen) is able to induce a potent transcriptional defense response in *C. elegans*, culminating in the induction of antimicrobial peptides by cells in the hypodermis. However, what is it precisely about mechanical damage itself that turns on these antimicrobial peptides? A recent study shed light on the mechanism: disruption of epidermal architecture, specifically, the disruption of the apical *C. elegans* hemidesmosome receptor MUP-4, induces the expression of antimicrobial peptides in a spatially-restricted fashion via the STAT family protein STA-2, a finding re-

capitulated in mammalian cells[241]. Moreover, extensive wounding in the epidermis is able to bypass the PMK-1 p38 MAPK cascade to directly activate STA-2 and upregulate antimicrobial peptides.

1.2.3 Perception of disrupted cellular physiology

One theory, which is supported by a growing body of evidence, is the idea of host surveillance of cellular functions. This point was first supported by the observation that feeding *C. elegans* RNAi directed against an essential gene (*e.g.*, *spg-7*, a mitochondrial AAA metalloprotease) recapitulated a behavior observed when these worms fed on a xenobiotic targeting the same gene product or organelle (*e.g.*, antimycin, an inhibitor of mitochondrial electron transport), called "avoidance behavior"[131]. This behavior, which will be described in more detail in Section 1.3.3, refers to the learned ability of *C. elegans* to avoid noxious food sources. In later studies, it was demonstrated that *C. elegans* responds to mitochondrial dysfunction though the activation of repair and drug detoxification pathways by monitoring ceramide biosynthesis, which has been known to accumulate on the outer membrane of the mitochondria after mitochondrial damage, such as during apoptosis[110].

Mitochondrial activity, however, is not the only cellular function that is monitored by the host. In independent studies, it was also discovered that a major gene signature of *P. aeruginosa* infection of *C. elegans* was the result of the host response to the disruption of one core cellular process, protein synthesis, by the *P. aeruginosa* ribotoxin ToxA, which ribosylates elongation factor-2[125]. Wild-type worms are resistant to ToxA-expressing *E. coli*, but worms deficient in the PMK-1, FSHR-1, or ZIP-2 pathways are sensitive, suggesting that immune pathways are required for defense against ribotoxin. This finding provides evidence supporting the significant overlap in immune signaling pathways and pathways that sense disruption of cellular physiology such as protein translation, or that the repair response to disruption of cellular physiology involves immune pathways.

Further evidence for innate immunity being an integrator of cell-restricted damage and systemic stress resistance comes from studies demonstrating that environmental stress can be detected in a tissue-specific manner by the activation of the DNA damage response in *C. elegans* germ cells. Be-

cause cells in the *C. elegans* germline, unlike the soma, undergo mitosis and meiosis, DNA checkpoints are critical for promoting DNA repair and preventing abnormal cell division. It was demonstrated that germline DNA damage activates the ERK1/2 homolog MPK-1, which induces putative antimicrobial peptides in the germline[35]. Additionally, MPK-1 activation triggers enhanced proteostasis in somatic tissues through the activation of the ubiquitin proteasome system, conferring systemic stress resistance, specifically to heat shock and oxidative stress, in the soma. This study illustrates that cells can communicate the presence of a stress in a cell non-autonomous manner in order to promote adaptation and survival (here, in a hormetic manner) and in particular, implicates the *C. elegans* germline as a sensor for stresses that then relays a protective signal to the soma.

Collectively, these results support a model for host surveillance of core cellular processes, such as protein translation. Impairment of these cellular functions appears to be perceived as a potentially virulent microbial attack, a concept that fits well with ideas about patterns of pathogenesis.

1.3 Immune effector mechanisms in *C. elegans*

In mammals, the innate immune system is the first line of defense that acts immediately after infection to clear the pathogen, or failing that, to hold the pathogen in check until an adaptive immune response develops. Invertebrates, however, do not have the advantage of "buying time," as they do not possess an adaptive immune system. Instead, they must harness all possible effector mechanisms to detect and destroy pathogens. Furthermore, in the case of *C. elegans*, it must accomplish this in the absence of any circulatory system, cytokines, or phagocytes – even the complement cascade is absent in *C. elegans*. I will next discuss the ancient immune effectors that are at play in the *C. elegans* immune response.

1.3.1 Antimicrobial effectors

From transcriptional profiling studies of different pathogen infections in *C. elegans*, it appears that the activation of PMK-1 upregulates at least three groups of putative secreted immune effectors, the

first of which is comprised of C-type lectin domain-containing proteins, which are secreted into the intestinal lumen in high levels. While it is thought that these proteins bind carbohydrates on the bacterial surface, there has been no definitive biochemical evidence to confirm that intestinally-secreted C-type lectins kill or impair bacteria. As C-type lectins are part of a highly expanded protein family in *C. elegans*, it has been speculated that the membrane-associated C-type lectins may act as pattern recognition receptors. Indeed, this is the case for the vertebrate immune system. For instance, the C-type lectin Dectin-1 serves as a fungal pattern recognition receptor in mammals[181]. Mannose-binding protein is another vertebrate C-type lectin that can bind and target bacterial pathogens for lysis through complement fixation, or simply act to opsonize bacterial pathogens for phagocytosis[219,226]. While C-type lectin receptors are often endocytic, they may also be capable of signaling; in *C. elegans*, it is possible that upon binding of a bacterial ligand, *C. elegans* C-type lectin receptors can signal through MAP kinases to induce immune effectors[30,220]. It is interesting to note that some of the same lectins in *C. elegans* are highly upregulated in response to several different pathogens, whereas some lectins appear to be relatively pathogen-specific[79]. This may suggest that these molecules may contribute to pathogen specificity of the *C. elegans* defense response.

A second group of PMK-1 secreted immune effectors are proteins containing a CUB domain, a conserved protein sequence common to a diverse group of primarily extracellular proteins and some plasma membrane-associated proteins, of which some are proteases[33]. The term CUB stands for C1r/C1s complement, embryonic sea urchin protein Uegf, bone morphogenetic protein 1 Bmp1, alluding to its evolutionary conservation. While they are typically thought to be developmentally regulated, it is possible that these proteins are upregulated during infection to bind and cleave proteins on the bacterial cell wall.

A third group of putative secreted immune effectors regulated by PMK-1 are antimicrobial peptides, which may be functionally similar to mammalian antimicrobial peptides, despite their lack of structural similarity. The *C. elegans* genome contains a number of different classes of antimicrobial peptides, including mollusk defensin/mycitin-like peptides (ABF-1 to ABF-6), which are upregu-

lated in response to *Salmonella typhimurium*, and neuropeptide-like proteins and caenacins, which are upregulated rapidly in response to fungal infection. It is possible that these antimicrobial peptides can directly kill bacteria and fungi by targeting their cell walls, or even penetrate into the cell to bind essential intracellular molecules[212].

C. elegans also possesses a number of other evolutionarily-ancient proteins that are thought to have direct microbicidal effects. One of these families of proteins are the caenopores, which contain a saposin domain that is also shared by the NK-lysin and granulysin proteins in mammals[180]. Two caenopores, SPP-1 and SPP-5, were shown to have bactericidal activity; furthermore, the mechanism by which SPP-5 was shown to kill bacteria was through permeabilizing the bacterial membrane[8]. Additionally, *C. elegans* lysozymes – some of which are related to those of protists or other invertebrate metazoans – have also been found to be upregulated upon bacterial infection[122,145,151]. Like many of the putative *C. elegans* immune effectors, the precise function of the lysozymes are not clear; it may be that, like the lysozymes in mammals, these enzymes act by damaging bacterial cell walls, hydrolyzing residues in bacterial peptidoglycan or fungal chitodextrins[127].

Whatever is the case, lysozymes, along with many of these other putative immune effectors, have undergone vast gene duplication in nematodes, suggesting not only their importance by evolutionary selection, but also the need for the functional diversification of these potential antimicrobial factors.

1.3.2 Xenobiotic detoxification response

Every organism will encounter compounds, exogenous or endogenous, that need to be metabolized and excreted. *C. elegans*, in its natural environment, must wade through and ingest a heterogenous slurry of microbial food. It is nearly inevitable, therefore, that *C. elegans* will eventually ingest pathogenic bacteria or fungi, or toxic molecules and xenobiotics, which will inflict cellular damage to the host. Unlike other metazoans, damaged cells in *C. elegans* cannot be replaced by a wound healing response, as all somatic cells in *C. elegans* are post-mitotic and non-renewing. Instead, *C. elegans* cells must repair their damaged biomolecules through the repair of DNA, proteins and lipids.

Detoxification is the most direct response a host can direct against xenobiotics. For this reason, *C. elegans* has evolved a robust, tripartite xenobiotic detoxification response to metabolize offending toxins. This vigorous stress response program consists of the participation of superoxide dismutases (SODs), heat shock proteins, cytochrome p450s, UDP-glucuronosyltransferases (UGTs), short-chain dehydrogenase/reductases (SDRs), and glutathione-S-transferases (GSTs), many of which are regulated by the insulin signaling pathway in *C. elegans*[80].

In the phase 1 response, host enzymes catalyze the addition of functional groups such as hydroxyls to the foreign molecule. This process progressively renders lipophilic xenobiotics water soluble, a characteristic critical for excretion, and introduces chemical groups that make them amenable to further metabolism. The CYPs, of which there are at least 86 in *C. elegans*, are the principal phase 1 enzymes and comprise a superfamily of heme-containing mono-oxygenases[195]. Additionally, the *C. elegans* genome contains 68 SDRs, such as alcohol dehydrogenases, which reside in both the smooth ER and cytosol and catalyze the reduction of carbonyl groups in aldehydes and ketones. Next, in phase 2, charged species (*e.g.*, glutathione and sugars) are conjugated to these altered molecules to further increase solubility, a step carried out by the GSTs and UGTs, of which there are 46 and 71 respectively in *C. elegans*. Finally, in phase 3, these solubilized metabolites are excreted by multi-drug efflux pumps, such as ATP-binding cassette (ABC) transporters[210]. As the *C. elegans* genome contains 60 proteins likely to be ABC transporters, this makes them the largest family of transporters in the *C. elegans* genome.

Because the detoxification program is energetically costly for the organism, it is only induced when needed in response to environmental stimuli. Furthermore, not all the detoxification enzymes encoded within the genome are induced at once[106]; instead, toxin-specific detoxification programs are switched on. This is achieved in part through the activation of DAF-2 as well as the transcriptional regulator SKN-1, which controls the induction of phase II detoxification genes (GSTs, UGTs) and oxidative stress-responsive genes (comprising major antioxidants such as catalases and superoxide dismutases)[80].

How exactly are xenobiotics perceived by *C. elegans* so that the detoxification response can be induced in a toxin-specific manner? One possibility is through perception by a large number of nuclear hormone receptors (NHRs). The *C. elegans* genome contains 284 NHRs – perhaps a testament to their importance in evolution – which regulate gene expression in response to many cues, including developmental, environmental, and nutritional signals; as a result, NHRs integrate numerous inputs and increase the modularity of the resulting transcriptional response [195]. This has already been observed in mammals, where NHRs have been found to play a central role in the regulation of the response to xenobiotics in mammals. In the mammalian liver, two well-characterized "xenosensing" NHRs, the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) family, have been shown to bind to their transactivating ligand, a xenobiotic, and subsequently trigger the expression of genes that encode metabolic enzymes (phase 1 and 2) and multi-drug efflux pumps (phase 3). The aryl hydrocarbon receptor (AHR), an NHR that binds aromatic hydrocarbons, has also received much attention recently from mammalian immunologists, as it appears to influence immunity at barrier sites [202].

Nuclear hormone family members may very well be critical in the recognition of a set of xenobiotics, but could this entire family of proteins be responsible for the perception and response to *all* xenobiotics? Given how many chemically and structurally different xenobiotics can be generated in nature, it is unlikely that each toxin is recognized by a different receptor in a "one receptor, one ligand" model, as space in the genome would certainly run out. The number of receptors, even in the *C. elegans* genome, is still too limited to be able to detect the inestimable number of possible xenobiotics. For this reason, the concept of cellular surveillance of essential processes, discussed previously, is very appealing. It would allow the worm to distinguish toxic xenobiotics from otherwise benign enviromental constituents.

1.3.3 Behavioral avoidance of pathogens

The well-documented "microbial aversion behavior" in *C. elegans* supports the existence of mechanisms that can recognize either MAMPs or DAMPs in a manner that is ultimately sensed by the worm nervous system to mediate avoidance[129]. While it is possible that recognition of a specific noxious agent derived from a pathogen could exclusively trigger avoidance behavior in *C. elegans* without concomitant immune effector mechanisms, *C. elegans* is likely to recognize a range of MAMPs concomitantly expressed in such pathogens, many of which are capable of triggering immune effector responses. Avoidance of *E. coli* expressing RNAi against genes essential for *C. elegans* is well documented[131], highlighting the role of DAMPs in the avoidance response.

Of the 959 somatic cells in the *C. elegans* hermaphrodite, 302 are neurons, 60 of which are ciliated neurons that comprise the primary sensory system and respond to stimuli – chemical, thermal or mechanical – and trigger many different behavioral processes and developmental decisions. Additionally, these chemosensory neurons express over a thousand G protein-coupled receptors, which may act as chemoreceptors that sense a smorgasbord of environmental inputs. Thus it is no wonder that *C. elegans* is highly equipped at discriminating between bacterial species, even preferring non-pathogenic bacteria. *C. elegans* appears to be capable of an "aversive learning response". In experiments presenting a choice between a lawn of pathogenic bacterium (such as *Serratia marcescens* or *P. aeruginosa*), or a lawn of non-pathogenic *E. coli*, nematodes initially prefer the pathogenic bacterial lawn, only to eventually desert it permanently for the *E. coli* lawn[16,62,164,240].

Similar observations have been made when worms have been allowed to feed on bacterial RNAi clones against genes essential for *C. elegans* (such as genes encoding actin, key mitochondrial proteins, or the proteasome) – these worms also flee the lawn[131]. From these studies, it became apparent that in *C. elegans*, cellular stress responses are coupled to neuroendocrine pathways through the signaling of KGB-1, a Jnk MAPK. In fact, in the case of *S. marcescens*, the molecular mechanism of sensation has been deciphered: *C. elegans* is able to detect and avoid the lawn through the sensa-

tion of one molecule, the cyclic lipodepsipentapeptide serrawettin W2, which acts on the two AWB chemosensory neurons of *C. elegans* to mediate avoidance [164]. In an independent study, phenazine-1-carboxamide and pyochelin, two secondary metabolites of *P. aeruginosa*, were found to activate a G-protein-signaling pathway in the ASJ chemosensory neuron pair that induced the expression of DAF-7 (homolog of TGF-β) [130]. DAF-7 was then shown to activate a canonical TGF-β signaling pathway in nearby interneurons to ultimately promote avoidance of *P. aeruginosa*.

These experiments not only demonstrate the surprisingly fine-tuned capability of worms for olfactory aversive learning, but also the worm's ability to integrate these many sensory inputs from their food – perhaps related to sensation of cellular damage [110,131], nutritional quality [196], oxygen concentration [14,204], texture [174], and of course, odors [19,56,235] – into the avoidance response, a fairly complex behavior for a small nematode [129].

1.4 Significance of this thesis

In the broadest sense, an immune system can be thought of as the body's mechanisms that protect the organism from noxious agents in the environment. These include chemical toxins or xenobiotics, pathogenic viruses and parasitic microbes. The immune system of a worm – and perhaps all metazoans – is organized in terms of (i) sensory pathways that can monitor "danger" both in the environment and in terms of cellular damage within the organism, and (ii) effector pathways that coordinate appropriate responses directed against the offending noxious agents such as secretion of antimicrobial peptides, xenobiotic metabolism and ultimately (iii) cellular repair and behavioral adaptation through avoidance responses, all of which contribute to increased survival and protection from pathology (Figure 1.3). There is a growing appreciation for the complex interactions between these three elements.

In this thesis, I have explored these interactions in *Enterococcus* infection models of *C. elegans* in an unbiased manner using using the toolbox available to a *C. elegans* experimentalist. Prior to the initiation of the studies described in this thesis, it was understood that damage to the host is a general

mechanism by which pathogenic organisms ensure infection and sometimes destruction of the host's cells. However, the mechanisms by which bacteria mediate direct or indirect damage to their hosts were still poorly understood. In bacterial infections of *C. elegans*, it was observed that the magnitude of the host defense response to *P. aeruginosa* correlates with the degree of virulence of infective bacteria, but the factors that triggered the intestinal defense response in *C. elegans*, and the effects of infection on the host, remained elusive. Furthermore, regarding enterococcal infection of *C. elegans*, little was known about the biology of the infection, despite the fact that a chemical screen for anti-infectives that rescued *E. faecalis*-infected *C. elegans* had already been performed.

In Chapter 3, I use the model host *C. elegans* to extensively characterize the *E. faecalis* infection, a pathogen known to cause a lethal infection in nematodes. I demonstrate using ultrastructural imaging that *E. faecalis* strain MMH594 proliferates in the *C. elegans* intestine, causing rapid distention even early in infection, but that this occurs in the absence of obvious host damage. Using whole-genome transcriptional profiling, I demonstrate that *C. elegans* mounts a defense response, driven primarily by MAMPs and not damage-associated molecular patterns. The *C. elegans* defense response, which is dependent upon immune signaling pathways, shares a common gene signature with other Gram-positive infection signatures.

In Chapter 4, I investigate the role of the *C. elegans* immune system in defense against *E. faecium* and find that like *E. faecalis*, *E. faecium* does not cause evident destruction of host cells. I demonstrate that while wild-type *C. elegans* are able to resist *E. faecium* infection and show no defect in lifespan, *C. elegans* that are deficient in *pmk-1*, *fshr-1*, or *bar-1* are hypersusceptible to *E. faecium* infection, suggesting that *E. faecium* is indeed a pathogen in *C. elegans*, and that an active host defense response is required to keep the *E. faecium* infection at bay. Using whole-genome transcriptional profiling, I show that colonization of the *C. elegans* intestine triggers a rapid transcriptional defense response dependent upon immune signaling pathways, similar to what has been observed in an *E. faecalis* infection. However, unlike the *C. elegans* host response to *E. faecalis* infection, the response to *E. faecium* is additionally dependent upon stress response signaling pathways. Specifically, a key set of

host effectors is regulated by the *C. elegans* SKN-1 and KGB-1 stress response pathways SKN-1 and KGB-1 and the *C. elegans* small RNA processing pathways, indicating potential cross-talk between the pathways responsible for pathogen resistance and stress resistance.

It is well-known from studies of the mammalian immune system that immune effector mechanisms are tightly linked to cellular repair processes, as uncontrolled activation of immune effector mechanisms can be damaging to the organism. I have demonstrated that the signaling pathways that sense the pathogen itself and those that sense cellular damage may be closely interlinked as described in Chapters 3 and 4 of this thesis. The results of this thesis may have parallels to concepts in mammalian immunology, and may inform therapeutic strategies as such interplay between these closely related physiological processes contributes to the pathophysiology of several chronic human diseases, such as sterile inflammation, chronic infections, or chronic autoimmune disease.

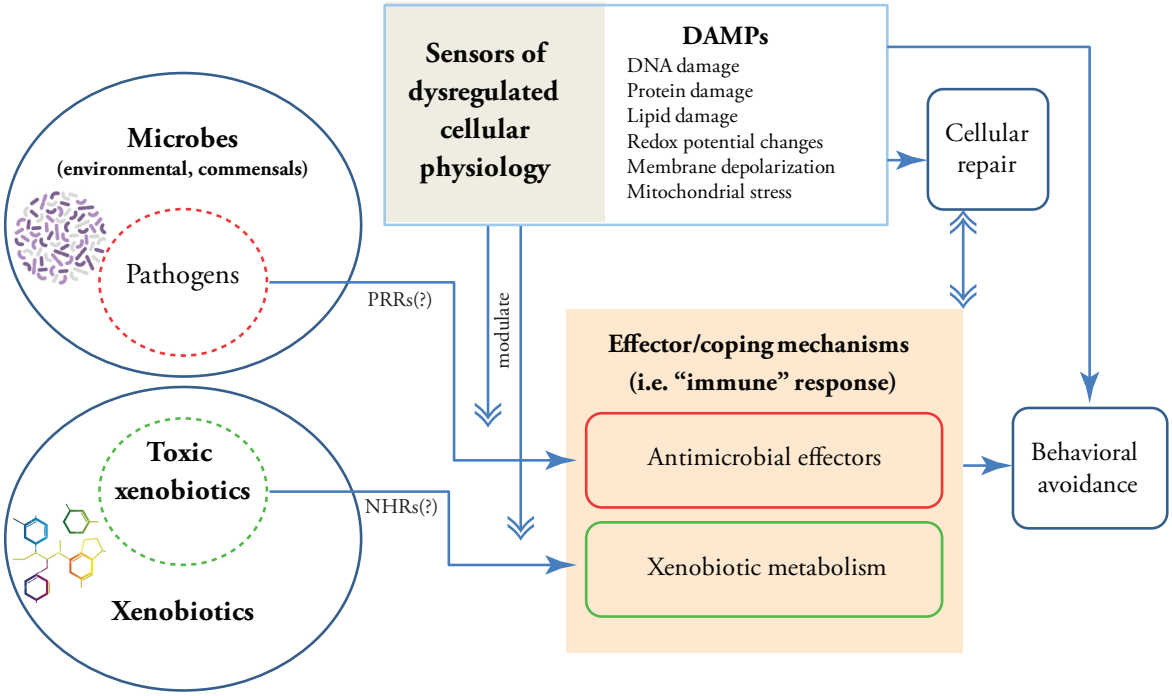


Figure 1.3: Integrated view of the immune system of a simple model organism. *C. elegans*, like any other organism, lives in a world that is surrounded by microbes and xenobiotics. As in vertebrates, receptors such as PRRs and NHRs may sense the presence of microbes and xenobiotics respectively. Concomitant sensing of host damage may modulate PRR and NHR signaling pathways, thereby alerting the host to the presence of a pathogenic microbe or a toxic xenobiotic. These signaling pathways, aided by extensive crosstalk among them, coordinate a multi-faceted host response to an infection involving the anti-microbial effector response, xenobiotic metabolism, cellular repair mechanisms, and in some cases, behavioral avoidance.

Gentlemen, it is the microbes who will have the last word.

Louis Pasteur

2

Enterococcus infection biology: lessons from invertebrate host models

Enterococci are a widely dispersed group of bacteria and more than 40 species have been described[46]. Two enterococcal species, *E. faecium* and *E. faecalis*, are commonly found among the commensal microflora in the human gut and typically do not cause disease. However, they can be important agents of human disease, especially when they infect extra-intestinal sites. The first disease associated with *Enterococcus* was a case of infective endocarditis in 1899[116]. *Enterococci*

This chapter is based on a review co-authored with Fred Ausubel for the Korean Society of Microbiology and has been published in near identical form in the Journal of Microbiology in 2014[239].

are currently the third most common nosocomial pathogen (12% of all hospital infections) causing urinary tract and wound infections, infective endocarditis, endophthalmitis, and peritonitis, which are often complicated by antibiotic drug resistance[133]. The majority of human enterococcal infections are caused by *E. faecalis* (isolated from 80-90% infections), with *E. faecium* comprising most of the remainder (10-15%)[85,87]. It is important to understand the biology of enterococcal infections to combat the rising rates of nosocomial infections. Within the past 25 years, many important insights have been gained into enterococcal infection biology from the viewpoint of host-pathogen interactions. In this chapter, I will focus on the insights gleaned from invertebrate infection models that have revealed how enterococcal species have become such a successful opportunistic, nosocomial pathogen, as well as the mechanisms by which the host innate immune system defends itself against enterococcal infection.

2.1 Invertebrate models of *Enterococcus* infection

Two invertebrate organisms have been used most successfully as model hosts for the study of enterococcal pathogenesis and will be discussed at length in this chapter: the greater wax moth *Galleria mellonella* and the free-living bacteriovorous nematode *Caenorhabditis elegans* (Figure 2.1). Compared to *C. elegans*, *G. mellonella* has a relatively complex innate immune system that may correlate better with observations in the mammalian host. On the other hand, *C. elegans* is genetically tractable with many genetic and genomic tools are available, as well as an extensive worldwide community of scientists focused on all aspects of *C. elegans* biology. This facilitates the discovery of novel features of the host-pathogen interaction. The advantages and disadvantages of these two infection models will be discussed at length in this chapter. While a few preliminary studies of enterococcal infection have also been carried out using *Drosophila melanogaster* as a model host[22,211], there has been very limited follow-up on these experiments.

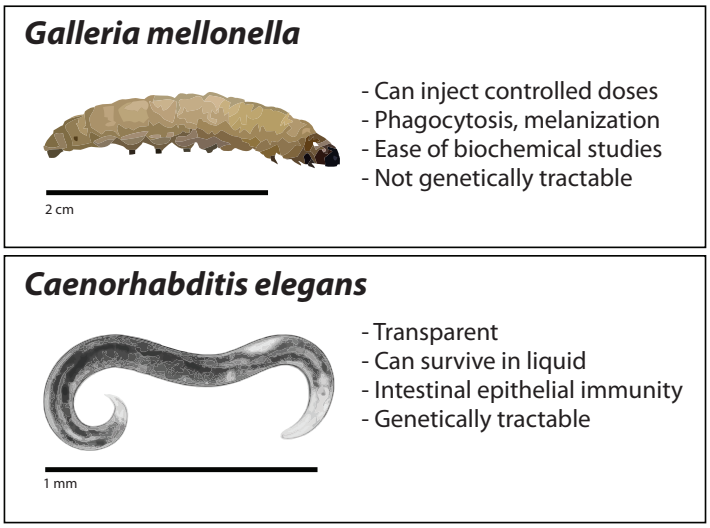


Figure 2.1: Key features of two invertebrate models

2.1.1 *Galleria mellonella* as a model host

Galleria mellonella (hereafter referred to as *Galleria*) larvae have been used to identify and characterize bacterial virulence factors that also cause disease in mammals, including humans, in part because of a good correlation between the role of bacterial virulence factors in the *Galleria* infection model and mice[81]. Studies using *Galleria* have identified several novel enterococcal virulence factors. While *Galleria* lacks a fully sequenced genome and genetic tractability, the *Galleria* infection model has been successfully used to biochemically identify enterococcal virulence factors in the insect hemolymph, as well as for studying novel protective responses in the host. The *Galleria* infection model of *Enterococcus* infection is also biologically relevant. The predominant organism colonizing the intestines of wild-caught *Galleria* larvae is the enterococcal species *E. hirae*, which is thought to have the potential to escape from the intestinal lumen to the body cavity during the larval to pupal transition, thereby inducing the host defenses of *Galleria*[11,32].

There are three main ways in which *Galleria* combat bacterial infections. First, circulating phagocytic hemocytes patrol the hemolymph, primed to recognize, engulf and sequester invading microbes. Second, upon detection of the pathogen within the inner cavity of the insect, proteolytic cascades can be quickly triggered, activating the melanization response (the synthesis and deposi-

tion of melanin to sequester pathogens at a wound site), followed by hemolymph coagulation, and opsonization[208]. The melanization response of insects is analogous to the formation of an abscess in mammals following an infection, in which neutrophils accumulate within a tissue to prevent the spreading of the pathogen. Third, *Galleria* is able to induce a menagerie of antimicrobial immune effectors such as lysozymes, as well as antimicrobial peptides, such as cecropins and moricins, which can be rapidly synthesized by the fat body[55]. The insect larval midgut also functionally parallels that of the mammalian intestine, as it is not only a major part of the digestive tract, but also regulates metabolism and the immune response[3].

An added benefit of using *Galleria* for pathogenesis studies is that the infections can be carried out at 37°C or higher, as *Galleria* is tolerant of higher temperatures, unlike *D. melanogaster* and *C. elegans* (maximum 25°C)[52]. This allows *Enterococcus* to grow optimally and at human body temperature for experimental studies. The larger size of the *Galleria* larva, compared to other invertebrate models, also allows it to be infected with larger, more controlled doses of the pathogen. In contrast, infection of *C. elegans* is carried out by allowing the animals to feed *ad libitum* on the pathogen, which does not control for dosage.

2.1.2 *C. elegans* as a model host

The small size of *C. elegans* and its ability to survive in liquid culture makes it amenable to cost-effective and rapid scaling-up for biochemical analysis[231], as well as for high-throughput screening of chemical libraries in whole-animals – a platform that would be impractical to implement in the case of *Galleria* larva (or even *Drosophila*), let alone vertebrates, due to their size[137,138]. Additionally, testing the effect of small volumes of rare natural compounds on infection biology is only currently possible using the worm infection model.

The ability of using *C. elegans* for the study of human pathogen infections was first demonstrated using the Gram-negative pathogens *P. aeruginosa* and *Salmonella enterica*. Subsequently, clinical isolates of Gram-positive bacterial pathogens, such as *E. faecalis* and *E. faecium*, were also shown to be

Table 2.1: Enterococcal virulence factors

Virulence factor	Description
Secreted toxic mediators	
GelE	Gelatinase; matrix metalloproteinase of broad proteolytic activity
SprE	Serine protease; homology to S. aureus
Cytolysin	Member of lantibiotic class of bacteriocins; able to lyse prokaryotic and eukaryotic cells
Surface adhesion molecules	
Esp	Enterococcal surface protein; adhesion to epithelium, biofilm formation and conjugation
Ace	Adhesin to collagen
Fss1-3	Adhesin to fibrinogen
Ebp	Adhesin to fibrinogen
Acm	Adhesin to collagen
Aggregation substance	Group of proteins encoded on pheromone-response, conjugative plasmids; role in transfer of antibiotic resistance genes and extracellular matrix proteins
Other toxic mediators	
Extracellular ROS	Superoxide and hydrogen peroxide; can induce oxidative stress in nearby host cells

able to infect *C. elegans*. Interestingly, while *E. faecalis* and *E. faecium* were demonstrated to accumulate in the intestine, only infection with *E. faecalis* caused a lethal infection, with the most virulent *E. faecalis* strains tested having an LT50 (the time taken to kill 50% of worms) of four days. This finding of *E. faecalis* infection being able to cause a lethal infection paved the way for further studies using *C. elegans* to screen for novel antimicrobials using *E. faecalis*-infected *C. elegans*, as well as to screen for previously unknown enterococcal virulence factors using an *E. faecalis* transposon library, the latter of which will be discussed in the next section.

2.2 Mechanisms of enterococcal virulence

There are a number of factors that contribute to the success of *E. faecalis* in establishing nosocomial infections. In addition to its ability to readily acquire antibiotic resistance and persist in harsh environments, *Enterococci* also have a substantial toolkit of virulence factors[4,44,94,187]. In the sections

below, I discuss the virulence factors and virulence-related processes that *Enterococcus* uses to establish an infection and the role that these factors in pathogenesis in *Galleria* and *C. elegans* (Figure 2.2).

2.2.1 Quorum sensing

Bacterial quorum sensing is the means by which bacteria are able to sense a species- or population-specific cell density via the production and secretion of small signal molecules. Quorum sensing can ultimately activate signaling transduction and thus modulate physiological behavior within the bacteria[160]. In Enterococci, quorum-sensing regulation is driven by the Fsr regulon, which is homologous to the Agr system of *Staphylococci*, perhaps the most well-described cyclic peptide-mediated quorum-sensing system among Gram-positive bacteria. The Agr quorum sensing system[57,99,114,150] and, to some extent, the Fsr system[12,20], have been discussed in several reviews. Briefly, the fsr quorum sensing system is comprised of four genes, fsrA (a response regulator), fsrB (a processing enzyme), fsrC (a sensor histidine kinase), and fsrD (a pro-peptide of the autoinducer)[142]. Following its production, the pro-peptide FsrD is processed and cyclized by FsrB to yield gelatinase biosynthesis activating pheromone (GBAP), an 11-amino acid cyclic peptide containing a lactone linkage[143]. GBAP then interacts with the sensor kinase FsrC, which leads to the phosphorylation of the response regulator FsrA, thereby inducing the transcription of not only the *fsrBC* operon, but also two nearby genes encoding secreted products, the serine protease SprE and the zinc-metalloprotease gelatinase GelE. The Fsr two-component regulatory system is a major regulator of virulence in *E. faecalis*, and disruption of the *fsr* operon impairs the transcription of these two downstream proteases encoding, as well as the development of biofilms[63,159]. *E. faecalis* fsrB mutants are attenuated in virulence in both the *C. elegans* and *Galleria* infection models, as well as mammalian infection models (mouse peritonitis and rabbit endophthalmitis)[49,198].

Does the host interfere with quorum sensing, and if so, how does the host counteract its effects? It has been proposed that, as quorum sensing is a feature of live and active bacteria, it may be targeted by

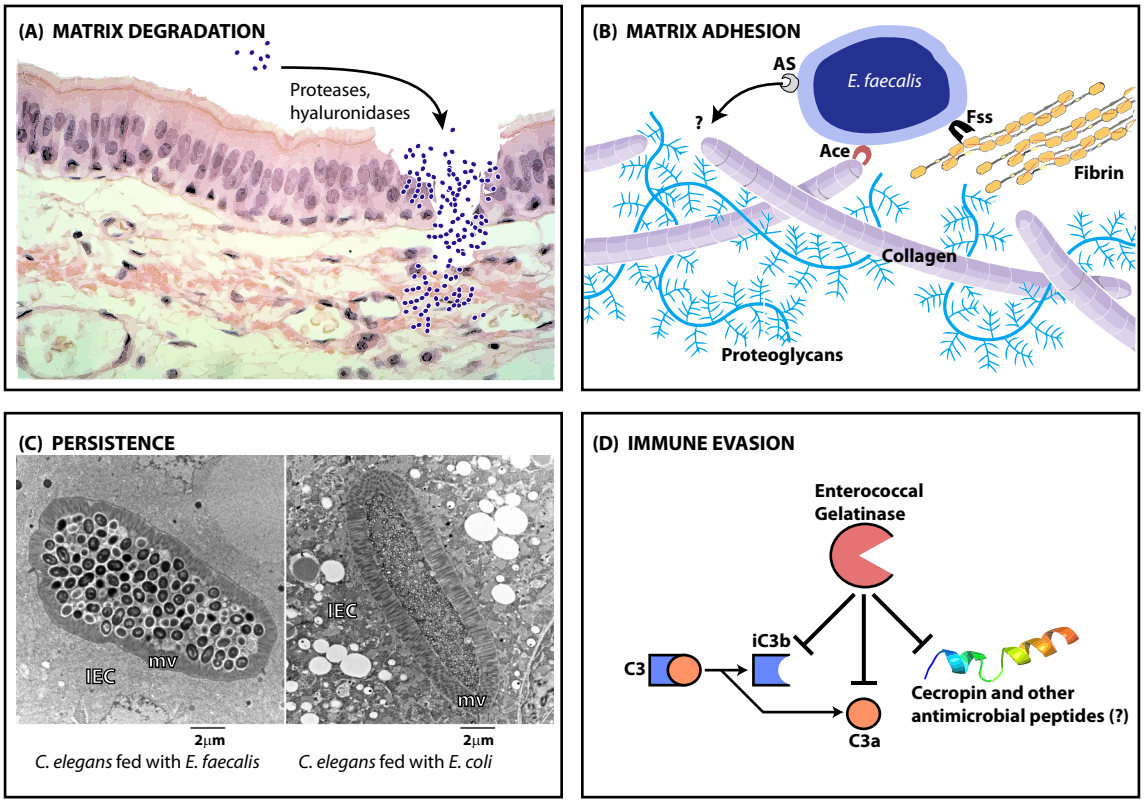


Figure 2.2: Mechanisms of enterococcal virulence. (A) Enterococci produce a number of enzymes (e.g., hyaluronidase, gelatinase, and serine proteases) that degrade the host extracellular matrix, which allow the bacteria to invade host tissues. **(B)** *E. faecalis* expresses several adhesins that allow it to bind the extracellular matrix and cell surface of the host. Aggregation substance (AS) facilitates the adherence of *E. faecalis* to extracellular matrix proteins. Ace and Fss are bacterial adhesins that bind collagen and fibrin, respectively. **(C)** Cross-section of the *C. elegans* intestinal lumen. After infection with *E. faecalis*, the *C. elegans* gut becomes distended with live enterococcal cells, forming a persistent infection in the worm. Most enterococcal cells are intact, with some even undergoing fission. In contrast, nearly all *E. coli* are degraded by the *C. elegans* grinder and intestine. Microvilli (mv) and intestinal epithelial cell (iec) are labeled. **(D)** Enterococcal gelatinase GelE aids in evading the immune system of both *G. mellonella* and humans. GelE degrades cecropin, an antimicrobial peptide induced early in infection. Additionally, GelE is able to hydrolyze human C3a and degrade C3b, two complement proteins generated by C3 activation. This inhibits opsonization, as well as the formation of the membrane attack complex, two important roles of the complement cascade.

the host immune system, which may try to recognize and inhibit the quorum sensing molecules[224]. In one study, a specific acyl homoserine lactone produced by *P. aeruginosa* (3OC12-HSL), but not other acyl-homoserine lactones (i.e., C12-HSL, C8-HSL, C4-HSL, or 2-amino-4-butyrolactone), was found to induce the chemotaxis of human polymorphonuclear neutrophils via a host receptor on the PMNs that directly bound 3OC12-HSL[245]. It is possible that the immune system has been selected to recognize these quorum-sensing auto-inducers as they are released from bacteria during the initial phases of biofilm formation, at a time in the infection when it may still be early enough for innate immune cells to stave off the infection. There may be a similar, corresponding interaction between enterococcal quorum sensing molecules and the host immune system. While there are no reports that this has been observed in *Galleria* or *C. elegans*, this interaction may be worthwhile to explore in the future.

2.2.2 Toxic mediators

Matrix-degrading enzymes

Proteases secreted by bacteria that degrade the host tissues serve to provide peptide nutrients for the bacteria. In the case of bacterial pathogens, the secretion of proteases is often concomitant with pathogenesis. Bacterial proteases damage the host through multiple mechanisms: direct destruction of both cells and extracellular matrix in the host tissue, indirect damage by proteolytic activation of bacterial matrix metalloproteinases or host proteolytic cascades[201], the processing of pathogenic virulence factors, and inhibition of host immunity[101].

Gelatinase (GelE) is a secreted zinc metalloproteinase that allows *E. faecalis* to degrade and invade host tissue by degrading the collagenous matrix and can interfere with host inflammatory processes by hydrolyzing a broad range of substrates including fibrinogen, fibrin, endothelin-1, bradykinin, human cathelicidin (LL-37), and complement components C3 and C3a[120,121,193,188,234]. GelE is also critical for biofilm development in *E. faecalis*, as it activates a peptidoglycan-degrading enzyme called autolysin, which is responsible for the release of extracellular DNA and the formation of a biofilm[214].

The formation of a biofilm allows *E. faecalis* to adhere to and survive on urethral catheters, urethral stents and cardiac valves, and enhances its survival in an antibiotic-rich environment[12,29,175,184,218]. Injection of purified GelE was found to be toxic to *Galleria* in a dose-dependent manner. *E. faecalis* mutants lacking GelE were also attenuated in virulence in Galleria. Although the insect does not contain any of the canonical targets of GelE like collagen, GelE was shown to directly hydrolyze a cecropin-like antimicrobial peptide in *Galleria*, analogous to its ability to degrade immune mediators in the human serum such as the complement component C3a. As C3a and C3a-derived peptides have broad antibacterial properties, the degradation of C3a by GelE may serve as an efficient way to cripple immune responses downstream of C3a. GelE is thus capable of subverting and inhibiting the immune system in both an insect and a mammalian infection model, in order to enhance bacterial survival in its host[193]. In contrast, injection of the purified enterococcal extracellular serine protease SprE did not cause any obvious damage to *Galleria*, although SprE was necessary for the full virulence of *E. faecalis* killing in *C. elegans*[197]. Like gelatinase, hyaluronidase is also a degradative enzyme; it hydrolyzes hyaluronic acid, contributing to host tissue damage and invasiveness of the infection. It is thought that disruption of host tissue may also allow other secreted bacterial factors to permeate the tissue, magnifying the damage[206]. The breakdown of host tissue products by hyaluronidase may also allow *Enterococcus* to access and metabolize the nutrients of its host[74,215].

Cytolysin

The enterococcal cytolysin, a secreted two-peptide lytic toxin, is related to the extensive group of lanthionine-containing bacteriocins produced by Gram-positive bacteria and is capable of lysing both prokaryotic and eukaryotic cells in response to quorum sensing signals[18]. The molecular properties of enterococcal cytolysin and its regulation have been well-studied[18,21,223]. One retrospective study found that of nearly 200 clinical *E. faecalis* isolates, 45% expressed cytolysin, which is encoded on a pheromone-responsive plasmid on a pathogenicity island[73]. The presence of cytolysin in a human infection has been reported to render the infection five times more lethal[73]. In the wild, *En-*

terococci have been isolated from *Galleria* larvae, and some of these isolates have been observed to exhibit lytic activity[83,84]. In the *C. elegans* infection model[47], as well as in a *Drosophila* oral ingestion model[22], noncytolytic *E. faecalis* strains were attenuated in virulence, although they still remained lethal to *C. elegans*.

2.2.3 Surface adhesion proteins

Enterococcal surface protein (Esp) is a surface adhesin that controls adherence to host tissues, some of which have been implicated in endocarditis. Following host heart tissue damage, *Enterococcus* can use its surface adhesins to adhere to the exposed extracellular matrix of the injured tissue[64]. The *esp* gene encodes a large LPxTG-anchored surface protein, which contributes to biofilm formation on abiotic surfaces. One study identified a putative surface antigen structurally related to Esp in vancomycin-resistant *E. faecalis* strain V583 called EF3314[24]. Mutations in EF3314 resulted in attenuated virulence in *C. elegans*, suggesting that this surface antigen may have targets in both mammals and nematodes.

Another potential adhesion of *E. faecalis* is the protein Ace, a collagen- and laminin-binding microbial surface component recognizing adhesive matrix molecules (MSCRAMM). Ace-specific antibodies have been found in the sera of patients with enterococcal infections; in particular, those patients with *E. faecalis* endocarditis, implying that the environment is an efficient trigger of Ace expression and that the expression of Ace is quite common[144]. Several *in vivo* environmental triggers, including high temperatures, the presence of collagen, and bile salts, can trigger transcription of *ace*. However, the first indication that Ace has a role greater than its binding to the extracellular matrix, came from *Galleria* infection studies, when *ace* mutants were found to be attenuated in virulence[102]. As collagen and laminin are both absent in insects, it is possible that Ace has other targets in *Galleria*, and perhaps in mammals as well.

Enterococcal aggregation substance (AS) is a pheromone-responsive, surface-bound, plasmid-encoded protein that promotes clumping of bacterial cells to facilitate plasmid exchange, as well

as adherence to intestinal epithelial cells by specifically binding extracellular matrix proteins. Additionally, AS allows *E. faecalis* to directly bind human neutrophils through complement receptor type 3 (CR3) binding[225]. Despite the close proximity of the bacteria to the neutrophils, these AS-expressing *E. faecalis* strains were resistant to neutrophil killing[171], and the oxidative burst (superoxide production) from the neutrophil may be responsible for causing collateral damage to neighboring tissues, without seriously damaging the *E. faecalis* cells. Interestingly, *E. faecalis* AS is not a virulence factor in the *C. elegans* killing assay[47], suggesting that the host target of AS is absent in *C. elegans*.

2.3 Novel concepts in host-pathogen interactions revealed by studies using invertebrates

2.3.1 Stress and its relation to virulence

Bacterial virulence is closely related to the ability of pathogens to adapt to different stresses. As bacteria traverse between the host and the outside environment during the course of the free-living and infection stages of their life cycles, they encounter myriad stressors, such as changing pH, temperature, and the presence of oxidative and osmotic stress. To establish a successful infection, the bacteria must be able to rapidly adapt to a changing environment, accompanied by vast changes in the expression of effectors and regulators of the stress response. These stress response genes are also required for bacterial virulence and have been explored in the *Galleria* model. Mutants in several stress-response genes were found to be attenuated in virulence, including the *clpB* gene, which encodes a Clp ATPase that is critical for promoting protein folding, as well as the assembly and degradation of proteins[25]. A second stress response gene that encodes methionine sulfoxide reductase A is also attenuated in *Galleria*, suggesting the importance of antioxidant repair enzymes in virulence[242]. Surprisingly, however, a deletion mutation in the *E. faecalis slyA* gene, which encodes a member of the MarR/SlyA family of bacterial transcription factors that is predicted to be a regulator of the virulence and stress responses, was shown to enhance virulence in the *Galleria* infection model, an unexpected

result for a transcriptional activator[132]. The enhanced virulence of the Δ*slyA* mutant was consistent with increased persistence in macrophage assays and *in vivo* mouse infections. Given these data, it is possible that the small subset of genes that are overexpressed in the Δ*slyA* mutant may be responsible for the enhanced virulence phenotype.

The "stringent response" is a bacterial response to nutritional stress (e.g. amino acid starvation) and is connected to enterococcal virulence. During the bacterial stringent response, two modified guanine nucleotides, guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), together known as (p)ppGpp, accumulate in the bacterial cell and act as "alarmones" or chemical messengers indicating stress, shutting down global transcription and selectively activating the transcription of genes regulating amino acid biosynthesis and stress-related survival. In *Enterococcus*, (p)ppGpp synthesis is catalyzed by two enzymes, RelA (the bifunctional synthetase/hydrolase of the alarmone) and RelQ (the monofunctional synthetase responsible for basal levels of alarmone during homeostasis). The alarmone (p)ppGpp was found to accumulate under vancomycin treatment and amino acid starvation. While *E. faecalis relA* and *relQ* single mutants did not show attenuated virulence in the *C. elegans* infection model, a *relAQ* double mutant, in which (p)ppGpp production is completed abrogated, was highly attenuated in its virulence[1]. This same result was also seen in *Galleria* infections[45] and the relAQ deficient strains also exhibited lower survival in macrophages *in vitro* than either the *relA* or *relQ* singly-deficient strains. These data support the model that basal levels of (p)ppGpp are required for full *E. faecalis* virulence in the infection of *C. elegans*, *G. mellonella* and mammalian cells. It is possible that the host innate immune system may recognize (p)ppGpp: the immune system is able to recognize the bacterial secondary messenger cyclic di-GMP as a MAMP[90,128,238], which, like (p)ppGpp, is also derived from the modification of a nucleotide.

2.3.2 Environmental persistence and biofilm production

The ability of *Enterococcus* to survive in a multitude of environments, from the human gut to an abiotic surface in a hospital, reflects its hardiness as a pathogen and capacity for environmental persistence. In addition to being thermo-tolerant (growing at 10-45°C), pH-tolerant (pH 4.8-9.6) and salt-resistant (up to 28% NaCl), *Enterococcus* is also able to survive extended desiccation[51], which is unusual for a bacterium that is incapable of sporulation. This may explain how *Enterococcus* is transferred from person to person in the hospital. *Enterococcus* may be able to enter a viable but non-culturable state when faced with nutrient-poor environments and may exist in this form on inanimate objects in the hospital, such as bedrails and medical station keyboards[65,112,124]. These intrinsic properties allow *Enterococcus* to flourish in a variety of environments, and may have been evolutionarily selected for by its need to survive in harsh environments, such as the intestinal tracts of animals.

The ability of *Enterococcus* to form a biofilm also allows *Enterococcus* to persist on abiotic surfaces. The biofilm protects the organisms from the host immune response, as well as antibiotics and antimicrobials, which leads to chronic enterococal infections that are difficult to treat[105]. Two potential *E. faecalis* regulators of biofilms may also control virulence in the *C. elegans* infection model. In screens for *E. faecalis* transposon mutants that were attenuated in killing *C. elegans*, two related, potential virulence factors were identified: the transcriptional repressor ScrR, and a gene that encodes a protein that is highly homologous to sucrose-6-phosphate hydrolases (ScrB) of other bacterial species and that is likely regulated by ScrR. This was intriguing, as sucrose utilization has been demonstrated to have a role in the formation of biofilms in dental caries and endocarditis by *Streptococcus mutans*, in which the sucrose catabolites are used to synthesize insoluble glucans to promote adherence to the tooth surface and heart valve[47,115].

V583 is a virulent strain and was also the first sequenced vancomycin-resistant *E. faecalis* strain. 25% of the genome of V583 contains mobile elements, including a pathogenicity-associated island[190, 156,191]. The oral *E. faecalis* isolate, OG1RF, is devoid of many genes related to pathogenicity and is

comparatively avirulent in humans. However both OG1RF and V583 have comparable virulence in the *C. elegans* infection model, even though OG1RF is believed to lack many genes thought to be required for virulence in humans[9,47]. One possibility is that the genes required for virulence in humans are not necessary to render *Enterococcus* pathogenic in *C. elegans*, while *E. faecalis* genes that promote commensal enterococcal colonization of the human gut in healthy individuals (seen in both OG1RF and V583) are sufficient to cause virulence in *C. elegans* infection. This latter conclusion is supported by studies of enterococcal metabolism. *Enterococcus* has an impressive flexibility to catabolize a smorgasbord of carbon sources[26,28,41]; *Enterococcus* is also able to use certain amino acids as energy and carbon sources[27,182]. Concomitantly, and almost paradoxically, *Enterococcus* also has a number of auxotrophies, requiring a number of amino acids (*e.g.*, valine, leucine, histidine, and tryptophan), as well as vitamins (*e.g.*, biotin, riboflavin and nicotinic acid)[51,141]. These features of enterococcal metabolism are conserved across both virulent and avirulent *E. faecalis* strains and may have been selected for by competition as a commensal organism in the mammalian GI tract[227].

Why are *E. faecalis* infections more common than *E. faecium* infections? A possible reason may be that *E. faecalis* is simply more abundant in the gastrointestinal tract than *E. faecium*; indeed, one study found that *E. faecalis* was on average 100 times more prevalent than *E. faecium*[149]. An interesting observation from enterococcal infection of *C. elegans* may hint at a reason for dominance of *E. faecalis*. Infection with *E. faecalis* or *E. faecium* rapidly distends the *C. elegans* intestine which fills up with live enterococcal cells. However, only *E. faecalis* results in a persistent and lethal infection in the worm, even with a small inoculum of *E. faecalis*. This may suggest that *E. faecalis* has an inherent ability to establish a persistent infection, perhaps contributing to its virulence. The unique characteristics of *E. faecalis* that allow it to form a persistent infection remain unknown. There does not appear to be a single virulence factor that contributes to the bulk of *E. faecalis* pathogenicity, as *E. faecalis* transposon mutant library screens have not picked up any single mutant that rendered the *E. faecalis* infection completely attenuated. The mechanism for *E. faecalis* persistence in *C. elegans* is unknown, but we speculate that *E. faecalis* may be better at competing for and establishing a protected niche for itself

in the *C. elegans* intestine.

2.3.3 Innate immune perception of enterococcal infection

As mentioned previously in Chapter 1, mammalian innate immunity relies upon the perception of a bacterial infection through pattern recognition receptors (PRRs), which recognize conserved microbial structures (e.g., flagellin, lipopolysaccharide, unmethylated CpG motifs) as microbe-associated molecular patterns (PAMPs). Recently, however, it has been proposed that the immune system may respond to MAMPs in the context of other signals, derived from either the host or the pathogen, that are only generated during a pathogenic infection, called patterns of pathogenesis[224].

These principles also apply to the innate immune system of invertebrates. In the case of *C. elegans*, several immune signaling pathways have been identified, although no specific MAMPs that activate *C. elegans* immunity have been discovered. Chemical genetics may be one approach to help identify novel and potentially conserved immune signaling pathways in *C. elegans*, which may ultimately lead to the identification of the most upstream innate immune receptors in *C. elegans*, and potentially, the identification of specific bacterial MAMPs recognized by such receptors. One chemical genetics study employed a liquid-based, high-throughput screen of small molecules and extracts to identify compounds that promoted the survival of *E. faecalis*-infected worms[137,138]. One compound selectively acted upon the *C. elegans* host via the activation of the well-studied and conserved p38 MAP kinase, PMK-1[168]. However, the precise biological target of this molecule is unknown. It is possible that this activating compound is modified in the host to generate a molecule resembling a pattern of pathogenesis or may activate signals that are also transduced by receptors that recognize patterns of pathogenesis. In addition to identifying novel immune activators, this approach can also be used to identify antimicrobials (which inhibit the growth of or kill bacteria) and inhibitors of bacterial virulence (which may not have an effect on bacterial survival, but may modulate bacterial physiologic behavior).

The DAF-2/DAF-16 (homologous to IGF-1 receptor and FOXO, respectively) insulin signal-

ing pathway is also involved in the *C. elegans* defense against *E. faecalis*. When the DAF-2 receptor is prevented from inhibiting DAF-16, genes involved in regulating lifespan, dauer formation, and pathogen and stress resistance are activated, leading to enhanced resistance to *E. faecalis* and other pathogens[48,140]. A number of DAF-16-regulated genes are necessary for the neutralization and detoxification of ROS, such as superoxide dismutases (which reduce superoxide to hydrogen peroxide) and catalases (which convert hydrogen peroxide to water and oxygen)[15]. This suggested that countering ROS is crucial component of an effective immune response; however, it was unknown whether the ROS was primarily generated by *E. faecalis*, or the host in response to pathogenic infection. It was later shown that *C. elegans* host intestinal cells, following infection by *E. faecalis*, generate extracellular ROS via the NADPH oxidase BLI-3, the *C. elegans* homolog of mammalian dual oxidase Duox1, which contributed to pathogen resistance[15]. The production of ROS, in tandem with the infection, activates a p38 MAPK PMK-1 cascade to induce the Nrf-family transcription factor SKN-1, which in turn upregulates stress and antioxidant responses during infection to protect against the collateral damage inflicted by endogenous ROS production[154,221]. This response may serve as an ancient form of the immunity, much like the oxidative burst observed in macrophages and neutrophils. It remains unknown, however, by what mechanism the dual oxidase is activated to eventually activate the immune response. How is *Enterococcus* infection perceived by the *C. elegans* host immune system? Is it through recognition of conserved structural motifs specific to pathogens, or by the recognition of perturbed cellular processes in the host? There is increasing evidence for the latter[31,125,131]. Host innate immune defense appears to be closely related to surveillance pathways that monitor core cellular activities (*e.g.*, organelle function, protein translation, *etc.*), which may also allow the host to perceive the presence of pathogens that produce virulence factors that subvert fundamental host functions.

2.3.4 *E. faecium* and its opportunistic traits

In comparison with other enterococcal species, strains of *E. durans* and *E. faecium* are either avirulent or weakly virulent in *G. mellonella* compared to *E. faecalis*. This intriguing interspecies difference in pathogenesis has also been observed in the *C. elegans* infection model, and may imply the presence of *E. faecalis*-specific virulence factors. It is not always the case, however, that *E. faecium* is avirulent. In studies with *C. elegans*, *E. faecium* that is grown under anaerobic conditions and then exposed to aerobic conditions, produces hydrogen peroxide, which is able to kill *C. elegans* rapidly within a few hours. The explanation behind this phenomenon is related to *E. faecium* being a facultative anaerobe relying on glycolysis and fermentation to generate energy. In *E. faecium*, lactic acid fermentation is favored under anaerobic conditions. However, under aerobic conditions, mixed acid fermentation takes place, where NADH oxidases use molecular oxygen as an electron acceptor to regenerate NAD^+ from NADH. In turn, in these aerobic conditions, molecular oxygen is reduced to hydrogen peroxide. The NADH peroxidase, which scavenges the hydrogen peroxide, is only expressed under aerobic conditions. Thus, the immediate exposure of anaerobically grown *E. faecium* to aerobic conditions allows for the production and accumulation of hydrogen peroxide, with levels of NADH peroxidase that are too low to scavenge efficiently[139]. It was also found that hydrogen peroxide production increased as aerobically-grown *E. faecium* entered the stationary phase. Taken together, the production of hydrogen peroxide may be one mechanism by which *E. faecium* is able to impede or kill its fellow competing bacteria, as well as damage host tissue or immune cells via oxidative stress.

Oxidative stress has also been speculated to act as a signal to promote the transition of *E. faecium* from a commensal to opportunistic state to render *E. faecium* more pathogenic. The *E. faecium* AsrR protein was identified as an oxidative stress sensor that uses cysteine oxidation to sense hydrogen peroxide. AsrR and its putative homologs are present in *E. faecium*, *E. gallinarum* and *E. casseliflavus*, but absent in *E. faecalis*. AsrR has been demonstrated to act as global repressor, inactivated by oxidative stress, leading to the expression of many genes involved in antibiotic resistance, biofilm for-

mation, and host colonization[103]. Furthermore, the Δ*asrR* mutant exhibited greater persistence in *Galleria* colonization and mouse systemic infection models, perhaps through the upregulation of two important adhesion-encoding genes, *acm* and *ecbA*. Thus, *E. faecium* may use oxidative stress as an important environmental signal to trigger a coordinated response to effectively colonize its host.

2.4 Conclusions

There remain several unanswered questions in *Enterococcus* infection biology, and many of these questions are especially tractable using invertebrate model systems, which allow for the genetic dissection of both the host and pathogen fronts. The use of invertebrate infection models will prove especially useful in recapitulating facets of *Enterococcus* infection that involve the interaction between *Enterococcus* and host tissues, rather than the interaction between *Enterococcus* and individual cells (*e.g.*, macrophages), which can be recapitulated in cell culture. By virtue of their small size, *C. elegans* allows high throughput genetic and chemical screens in whole-animal infections for novel virulence factors as well as anti-infective compounds. In addition, *C. elegans* is genetically tractable and can be used for forward- and reverse-genetic screens to identify host factors required for defense against *Enterococcus*. However, genetic tools are rapidly improving, and it will not be long before *Galleria* can also be manipulated genetically, perhaps through the use of the CRISPR-Cas9 system and other genome editing tools. The use of bacterial genetics tools such as Tn-seq, which utilizes next-generation sequencing for identifying transposon insertion mutants of interest, will also help elucidate unique features of *Enterococcus* virulence such as its ability to establish a persistent infection.

Pathogens lurking everywhere,
Go get 'em first, they never fight fair.

Shiv Pillai

3

Live and heat-killed *Enterococci* activate *C. elegans* host defense via known immune pathways

Intestinal epithelial cells are juxtaposed with ingested bacteria, of which there can be hundreds of thousands inside a single *C. elegans* animal. Upon perceiving a bacterial infection, these intestinal epithelial cells activate signaling pathways that culminate in the secretion of large amounts of antimicrobial factors. However, this process must be carefully regulated so that the defense responses are activated under appropriate conditions, as inappropriate activation can lead to wastage of organismal and cellular resources, cellular damage, or even host death. It is not fully

understood how intestinal epithelial cells perceive the presence of a pathogen, and furthermore, discriminate between pathogenic and non-pathogenic microbes. One pathogen detection mechanism is the detection of microbe-associated molecular patterns (MAMPs) – conserved structural motifs that are shared by nearly all bacteria. However, while much is known about the activation of mammalian, and even *Drosophila*, signaling pathways via MAMPs, little is known about whether *C. elegans* is able to perceive MAMPs. For a long time, it was assumed that *C. elegans* was blind to MAMPs, as it is a natural bacteriovore, and thus must be somewhat tolerized to most MAMPs, otherwise it would be perpetually expending its energy and resources to activate immune responses, which seems unlikely.

However, there were some indications that *C. elegans* might be able to perceive MAMPs for certain pathogens. In one example, *C. elegans* fed with heat-killed *S. aureus* were found to activate a transcriptional response similar to that of live *S. aureus*, suggesting that the host response to *S. aureus* is possibly mediated by MAMPs in a manner independent of Toll signaling [78]. This is in contrast to heat-killed *P. aeruginosa*, which fails to upregulate genes that are activated by live *P. aeruginosa*, suggesting that *P. aeruginosa* infection appears to be recognized via DAMPs [78]. Indeed, a major gene signature of *P. aeruginosa* infection of *C. elegans* is the result of the host response to the disruption of one core cellular process, protein synthesis, by the *P. aeruginosa* ribotoxin ToxA, which ribosylates elongation factor-2 [125]. With these results in mind, I investigated the mechanism of pathogen recognition by studying infection of *C. elegans* with two enterococcal species, *E. faecalis* and *E. faecium*.

The use of *C. elegans* for the study of human pathogen infections was first demonstrated using the Gram-negative pathogens *P. aeruginosa* and *Salmonella enterica*. Subsequently, clinical isolates of Gram-positive bacterial pathogens, such as *E. faecalis* and *E. faecium*, were also shown to be able to infect and kill *C. elegans*. While both enterococcal species accumulated at high levels in the intestine and were able to kill nematode eggs and hatchlings, only *E. faecalis* killed adult worms [47].

The most virulent *E. faecalis* strains tested had an LT50 (the time taken to kill 50% of worms) of four days. To gain a better understanding of the infection process, Garsin *et al.* undertook differential

interference contrast (DIC) microscopy of worms fed on different bacteria and found that worms fed on *E. coli* and *B. subtilis* had no visible intact bacteria present in the intestinal lumen. This was in stark contrast to the grossly distended lumens of worms fed on *E. faecalis* and *E. faecium*, which were full of intact bacteria. The authors then tested whether a small number of *E. faecalis* bacteria could kill *C. elegans* using mixed lawns in which the ratio of *E. faecalis* to *E. faecium* was varied, and showed that as the percentage of *E. faecalis* was decreased in the initial lawn inoculum, killing still occurred but there was a longer lag time before the worms were killed. Even when worms were allowed to feed on a mixed lawn of *E. faecalis* and *E. faecium* in a $1{:}10^3$ ratio for 12 hours, and then moved to a lawn of only *E. faecium*, the number of live *E. faecalis* bacteria in the nematode gut still increased over the course of a few days, from being undetectable to containing 10^4 c.f.u. per worm, just 60 hours after being moved to the *E. faecium* lawn. In these experiments, the number of *E. faecium* cells per worm remained approximately constant, with a stable population of about 10^5 *E. faecium* cells in the intestine. These results suggested that *E. faecalis* was not only proliferating in the worm intestine, but that it had established a persistent infection. A similar transfer experiment was carried out with two *E. faecium* strains, E003 and E007, which were plated at a starting ratio of $1{:}10^3$, respectively. Over time, the ratio of these two strains in the worm intestine never deviated from the initial ratio, implying that the high titer of *E. faecium* observed in the gut was not primarily due to persistent colonization.

Taking advantage of the ability of *E. faecalis* to persistently colonize *C. elegans*, a high throughput chemical screen was carried out to identify synthetic compounds and natural product extracts that promoted the survival of *E. faecalis*-infected worms[137,138]. Such a screen allowed for the discovery of compounds that would have otherwise displayed no antimicrobial activity in traditional *in vitro* experiments, such as pro-drugs, compounds that target bacterial functions important only for *in vivo* survival or virulence, or activators of innate immunity. Of the couple dozen compounds that were found to protect *C. elegans* from *E. faecalis* infection, one was shown to activate the PMK-1 p38 MAPK pathway, in addition to upregulating xenobiotic detoxification programs[168].

Aside from this, little was known about the biology of *E. faecalis* infection in *C. elegans*, as well as the

immune pathways that were required for the host response to *E. faecalis*. In this chapter, I characterize the pathogenesis of *E. faecalis* infection in *C. elegans* using ultrastructural imaging, whole-genome transcriptional profiling, and multiplexed gene expression analysis. I demonstrate that *C. elegans* mounts a defense response, driven primarily by MAMPs, that is dependent upon known immune signaling pathways, and which shares a common gene signature with other Gram-positive infection signatures.

3.1 Results

3.1.1 *E. faecalis* proliferates in and causes distention of the *C. elegans* intestine

Prior to our studies, *E. faecalis* and *E. faecium* had been shown to both distend and proliferate in the *C. elegans* intestine, though only infection of *C. elegans* with *E. faecalis* was lethal. To gain insight into the host cellular perturbations and cytopathology caused by *E. faecalis* infection of the *C. elegans* intestine, I employed transmission electron microscopy (TEM) to image the ultrastructure of N2 wild-type worms that were fed either *E. coli* (grown on NGM) or *E. faecalis* (grown on BHI) at 8, 24, and 48 hours after infection. It was necessary to grow *E. coli* and *E. faecalis* on NGM and BHI respectively, as *E. faecalis* is unable to grow on NGM, and *E. coli* grown on BHI decreases the lifespan of *C. elegans*.

In *E. coli*-fed *C. elegans* worms (Figure 3.1 A, C, and E), nearly all of the bacterial cells in the intestinal lumen are macerated, with only 1-3 intact *E. coli* bacterial cells visible on average in each section. From 8 to 48 hours post infection, the *E. coli*-fed worms display long, straight intestinal microvilli, anchored at their base into the terminal web. These data are consistent with prior studies where the intestinal contents of *E. coli*-fed *C. elegans* were extracted and enumerated, showing 10-100 live *E. coli* cells were recoverable from each worm. One plausible explanation for why *E. coli*-fed *C. elegans* have a low load of live intestinal bacteria is because of efficient grinding in the pharynx and digestion in the intestine. In *C. elegans*, bacteria that are ingested by *C. elegans* first pass through the pharygeal

grinder, an organ that mechanically crushes ingested food particles such as bacteria. Subsequently, these bacterial products enter the intestine, which secretes digestive enzymes into the lumen to break down the bacteria further. Thereafter, the processed bacterial products and nutrients are taken up by the intestinal cells.

In contrast to *E. coli*-fed worms, *E. faecalis*-infected worms were found to have largely intact bacteria packing and distending the intestine, to the point where the bacterial cells were in immediate and continued contact with the microvilli, almost appearing to compress them (Figure 3.1, B, D, F). However, while the apical microvilli remained intact, did not decrease in length, and looked generally healthy, even at 48 hours post infection, the basolateral surface of the intestinal cells lacked a smooth border and instead appeared undulatory. Additionally, beginning at 8 hours after infection, conspicuous linear, grainy structures in the cytoplasm of the intestinal cells, as well as some dehiscence of the terminal web from the luminal membrane is apparent. There is little observable difference in cytopathology between 8 and 48 hours post infection, although a possible increase in mitochondria can be seen in the images of the *E. faecalis*-infected worms at 24 and 48 hours after infection. Thus, while the *C. elegans* intestine becomes distended with proliferating pathogenic bacteria early in *E. faecalis* infection, this appears to occur in the absence of severe host cellular damage.

Ultrastructural imaging of *E. faecalis*-infected worms demonstrated that *E. faecalis* infection in *C. elegans* is vastly different from previously characterized infections of *C. elegans* with either *P. aeruginosa* (characterized by intestinal distention intracellular invasion from the intestine and arrested autophagosomes), or *S. aureus* (characterized by effacement of the microvilli and lysis of the intestinal cells)[78], supporting the notion that the mode of pathogenesis of *Enterococcus* is likely different from that of these other previously characterized bacterial pathogens.

3.1.2 The role of cytolysin in *E. faecalis* infection of *C. elegans*

Though ultrastructural imaging did not reveal any obvious cytopathological effects of the *E. faecalis* infection, prior studies have shown that several *E. faecalis* factors, including aggregation substance,

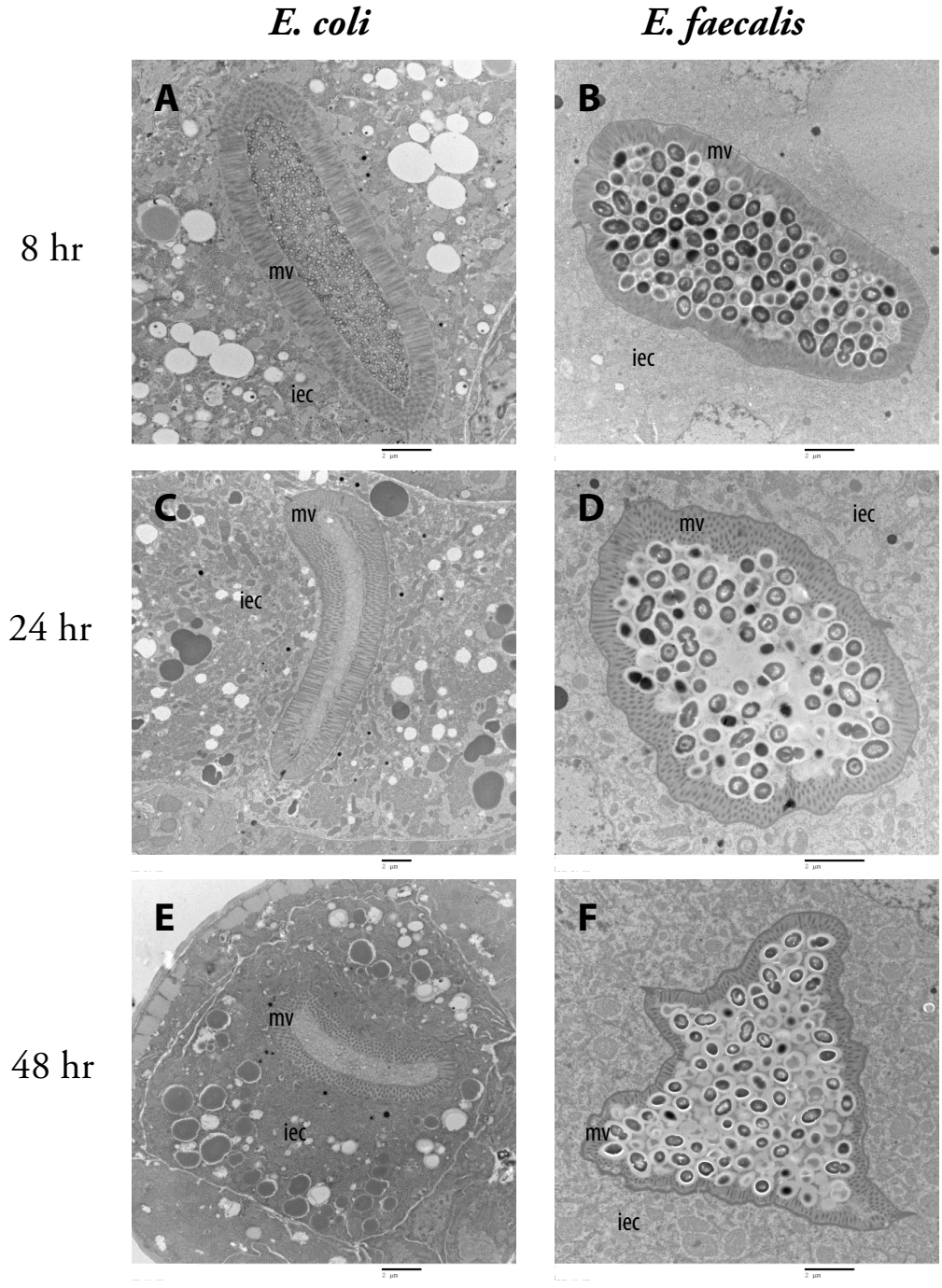


Figure 3.1: *E. faecalis* proliferates in and causes distention of the *C. elegans* intestine, but does not invade intracellularly or lyse intestinal cells. Transmission electron micrographs of transversal midbody sections of *C. elegans* feeding on non-pathogenic *E. coli* OP50 (A, C, E) or pathogenic *E. faecalis* MMH594 (B, D, E) at 8 hours (A, B), 24 hours (C, D) or 48 hours (E, F) post infection. The microvilli (mv) and cytoplasm (iec) of an intestinal epithelial cell are marked. Scale bar, 2 μm.

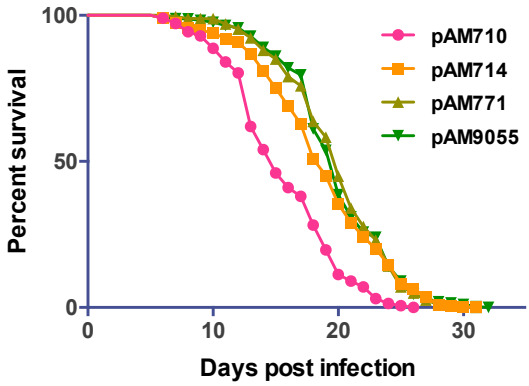
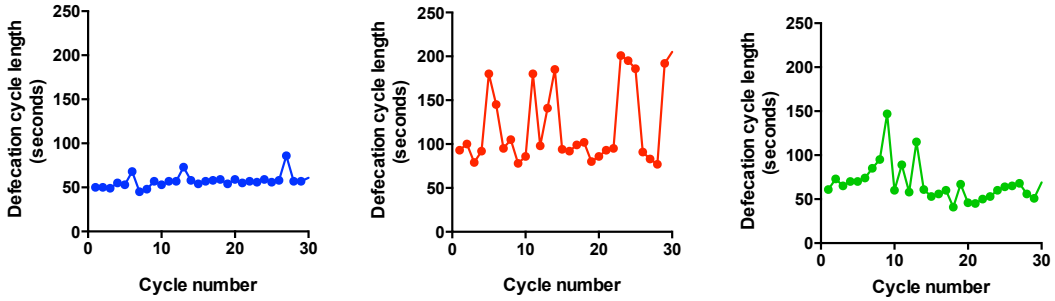


Figure 3.2: Infection of *C. elegans* with *E. faecalis* strains of varying hemolytic activity. *C. elegans fer-15;fem-1* L4 worms were infected with the following *E. faecalis* FA2-2 strains: pAM714(wild type), pAM710(hyperhemolytic), pAM771(CylL-deficient, non-hemolytic) and pAM9055(Cyl-A deficient, non-hemolytic). The difference in lifespan between pAM714 and pAM710 is statistically significant ($p < 1.0 \times 10^{-10}$). The enhanced resistance of worms to pAM771 and pAM9055 was of borderline significance ($p = 0.019$ and 0.029, respectively).

cytolysin, and the Fsr quorum sensing module, play a role in *C. elegans* lethality during *E. faecalis* infection[47]. To further probe the role of the *E. faecalis* cytolysin in infection of *C. elegans*, I infected sterile *fer-15;fem-1* mutants with each of four *E. faecalis* strains (FA2-2): wild-type *E. faecalis* strain (pAM714), CylL-deficient non-hemolytic (pAM771), Cyl-A deficient non-hemolytic (pAM9055), or a hyperhemolytic strain (pAM710) (Figure 3.2). The temperature-sensitive sterile *fer-15;fem-1* strain was used to avoid confounding effects of progeny in the survival assay.

While wild-type *E. faecalis* FA2-2 killed *C. elegans* with an LT50 of 18 days, the hyperhemolytic strain killed worms more rapidly, with an LT50 of 15 days. In contrast, worms infected with either of the non-hemolytic strains were killed with an LT50 of 19 days, with kinetics very similar to that of the wild-type *E. faecalis* strain. These results suggest that the cytolysin from *E. faecalis* may have a biological target in *C. elegans*, since overexpression of the cytolysin causes more rapid death. However, in the absence of the cytolysin, there may be compensatory virulence mechanisms that *E. faecalis* uses to kill the nematode, as the non-hemolytic strains have only a very modest decrease in pathogenicity.



(a) *E. coli* **(b)** *E. faecalis* **(c)** *E. faecium*

Figure 3.3: *E. faecalis* perturbs defecation cycle length in *C. elegans*. Defecation cycle lengths were recorded for N2 and *pmk-1* worms. A record of sequential defecation cycle periods in wild-type *C. elegans* fed on (A) *E. coli*, (B) *E. faecalis*, or (C) *E. faecium*. Variations in cycle length for *E. coli*-fed worms are minimal due to the rhythmic behavior. For the *Enterococcus*-infected worms, large fluctuations from cycle to cycle indicate a disruption in the rhythmic behavior. Length of defecation cycle is measured as the time between consecutive contractions of the posterior body wall muscle.

3.1.3 *E. faecalis* infection perturbs defecation

Because *E. faecalis* proliferates and distends the *C. elegans* intestine, even just 8 hours post infection, I was interested in identifying the physiological processes that might be perturbed by *E. faecalis* to allow for such efficient colonization. I hypothesized that defecation, previously shown to be compromised in some Gram-positive bacterial infections of *C. elegans*, may be one such process altered by *E. faecalis*. The posterior segment of the *C. elegans* intestine functions as the "pacemaker" of the defecation cycle, and I speculated that live *E. faecalis* growing in the intestine may be slowing down the rate of defecation in *C. elegans*, thereby allowing bacterial cells to accumulate[126]. To assess this, I examined *C. elegans* wild-type L4 larvae that had been fed *E. coli* (grown on NGM) or *E. faecalis*. Each defecation cycle in the *C. elegans* hermaphrodite begins with posterior body muscle contraction, which squeezes the anterior intestinal contents. After a short period of relaxation that allows the intestinal contents to flow towards the posterior, the ingested products are then concentrated near the anus and expelled.

In *C. elegans* fed *E. coli* (Figure 3.3a), I observed that defecation consistently occurred about every 50 seconds, similar to what has previously been reported for healthy *E. coli*-fed *C. elegans*[10]. In stark

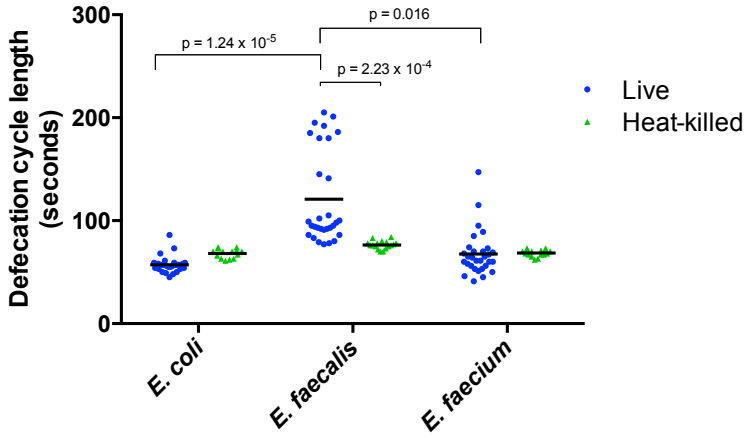


Figure 3.4: Defecation cycle lengths of wild-type N2 *C. elegans* fed live or heat-killed bacteria. A population of L4 worms were fed *E. coli*, *E. faecalis*, or *E. faecium*, either live or heat-killed, for 24 hours. Horizontal bars within the plotted points indicate the mean defecation cycle length. The differences between the live and heat-killed bacteria-fed *C. elegans* was significant for *E. coli*-fed worms ($p = 1.23 \times 10^{-5}$) and *E. faecalis*-fed worms ($p = 2.23 \times 10^{-4}$), but not for *E. faecium*-fed worms ($p = 0.84$). Statistical significance was calculated using an unpaired t-test and the Holm-Sidak method for multiple comparison correction.

contrast, *E. faecalis*-infected *C. elegans* displayed a highly irregular and abnormal defecation rhythm, with defecation cycles that ranged from 65 seconds to more than 3 minutes in length (Figure 3.3b). In studies with *C. elegans* feeding on *E. faecalis* expressing red fluorescent protein, I observed that even when these *C. elegans* worms attempted to expel their intestinal contents, they sometimes were unable to actually do so; infected worms expelled either less bacteria during each defecation compared to *E. coli* expressing red fluorescent protein, in some severe cases, little to no bacteria at all (data not shown).

E. faecium-infected *C. elegans* worms were examined next, as *E. faecium* is also able to distend and proliferate in the *C. elegans* intestine, but without compromising host survival. *E. faecium*-infected *C. elegans* exhibited a somewhat intermediate phenotype, with occasional cycle lengths above 100 seconds (Figure 3.3c, right). The majority of the sampled defecation cycle lengths hovered close to 50 seconds, similar to that of *E. coli*-fed *C. elegans*.

To assess whether a live, active infection of the *C. elegans* intestine was required to perturb defecation cycle length, I examined the defecation cycles of wild-type L4 *C. elegans* larvae fed for 24

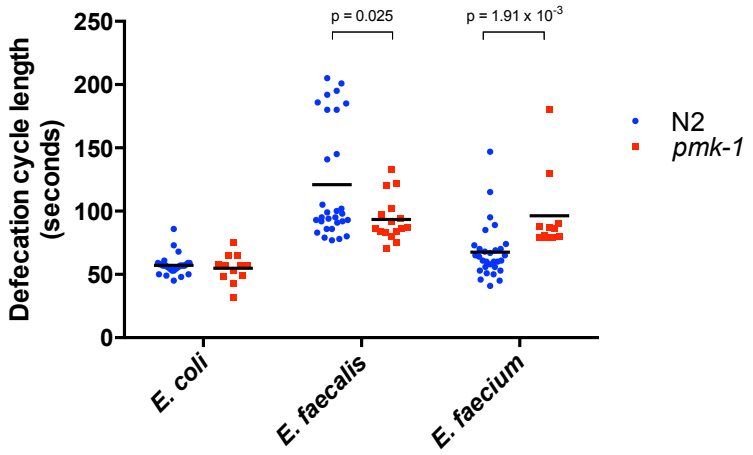


Figure 3.5: Defecation cycle lengths of N2 or *pmk-1 C. elegans* fed live bacteria. Defecation cycle lengths were recorded for N2 and *pmk-1* worms. Statistically significant differences were observed between N2 and *pmk-1* for *E. faecium* ($p = 1.91 \times 10^{-3}$), but not for worms fed on *E. coli* ($p = 0.450$); the difference between N2 and *pmk-1* was of borderline statistical significance for worms fed *E. faecalis* ($p = 0.025$). Statistical significance was calculated using an unpaired t-test and the Holm-Sidak method for multiple comparison correction.

hours on *E. coli*, *E. faecalis*, or *E. faecium*, either heat-killed or live. As observed in the previous experiment, worms infected with live *E. faecalis* showed a significantly higher average defecation cycle length than worms fed on *E. coli*, whereas worms fed on *E. faecium* only showed a modest increase in defecation cycle length (Figure 3.4). *C. elegans* fed on heat-killed bacteria, whether *E. coli*, *E. faecalis*, or *E. faecium*, had defecation cycles of similar lengths (means of 68.1, 76.5, and 68.6 seconds, respectively). The difference between live and heat-killed bacterial samples for the *C. elegans* fed on *E. faecalis* was statistically significant, suggesting that live *E. faecalis* impaired the normal defecation rhythm in *C. elegans*. *C. elegans* fed heat-killed *E. faecium* showed no statistical difference compared to *C. elegans* fed live *E. faecium*. These data also demonstrate that intestinal accumulation of bacteria was not sufficient to disrupt defecation rhythm, as *E. faecium*-infected worms did not have a severely compromised rhythmic behavior.

Given that an active, live *E. faecalis* infection was required to perturb the *C. elegans* defecation rhythm, I reasoned that a *C. elegans* mutant that lacked an intact immune system might show a heightened impairment in defecation rhythm, as these immunodeficient worms may have a higher level of

E. faecalis cells in their intestines, which could exacerbate intestinal stasis. I recorded the defecation cycle lengths 24 hours post infection of wild-type N2 and mutant *pmk-1* worms on live *E. coli*, *E. faecalis*, and *E. faecium* and found that while the presence of *pmk-1* had no effect on defecation cycle length in *E. coli*-fed worms, worms deficient in *pmk-1* showed longer defecation cycles when fed *E. faecium* (Figure 3.5).

Because both *E. faecalis* and *E. faecium* pack the *C. elegans* intestine but only *E. faecalis* kills the worms and causes an impairment in defecation rhythm, these results suggest that an *E. faecalis* infection actively perturbs the defecation cycle, rather than a defect in defecation being the primary factor affecting worm longevity. In Chapter 4, I show that *C. elegans pmk-1* mutants die prematurely when fed *E. faecium*, suggesting that *E. faecium* has the potential to be pathogenic, but is normally kept in check by the *C. elegans* immune response. Thus, the observation that *pmk-1* mutants exhibit an aberrant defecation cycle when fed *E. faecium* is consistent with the conclusion that the pathogenic process, including perhaps the extent and timing of intestinal packing, affects the defecation process. The difference between N2 and *pmk-1* worms fed *E. faecalis* was of borderline statistical significance; the absence of a large difference even though *pmk-1* mutants are more susceptible to *E. faecalis* (Figure 3.16) may be due to the fact that the number of *E. faecalis* cells reaches a maximum capacity in the *C. elegans* intestine at 24 hours post infection, and a deficiency in the PMK-1 pathway does not result in any further increase in *E. faecalis* cell numbers.

3.1.4 The *E. faecalis* infection gene signature

Given the high bacterial load of *E. faecalis* in the *C. elegans* intestine and *E. faecalis* bacterial cells pressed up so closely against the *C. elegans* intestinal epithelial cells, I hypothesized that *C. elegans* likely mounts a defense response to *E. faecalis* during the infection process. To characterize the *C. elegans* host response to *E. faecalis* infection, I undertook whole-genome transcriptional profiling of *E. faecalis*-infected *C. elegans* young adult animals, comparing the expression profile to *C. elegans* fed on heat-killed *E. coli*. Heat-killed *E. coli* was preferable to heat-killed *E. faecalis* as a control, since it

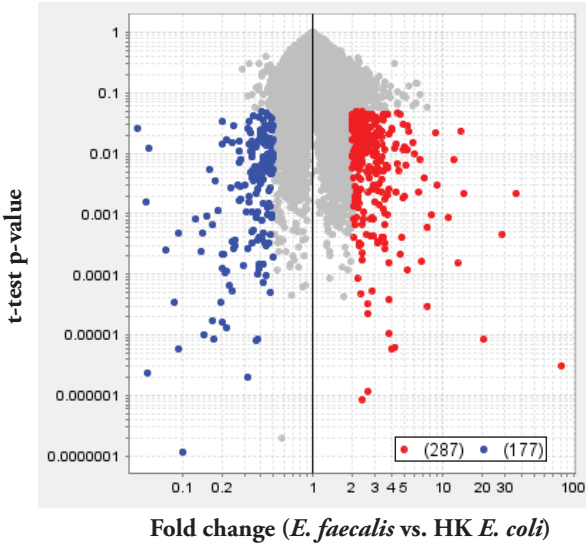


Figure 3.6: The *E. faecalis* infection gene signature. Volcano plot of *C. elegans* genes that were differentially regulated in *E. faecalis*-exposed versus heat-killed *E. coli*-exposed young adult worms. *E. faecalis* induces a rapid response 8 hours after infection. The *C. elegans* genome array contains 22,548 sequences. Highlighted in red and blue are the genes corresponding to the *E. faecalis* infection gene signature; colored points indicate genes with $>$ 2-fold change ($p < 0.05$).

was unclear whether heat-killed *E. faecalis* would activate a response similar to that of live *E. faecalis*, akin to what has been observed in studies of the *C. elegans* transcriptional response to heat-killed and live *S. aureus*[79]; if this were the case, the use of heat-killed *E. faecalis* as a baseline might reveal few differentially regulated genes.

I identified 287 genes differentially upregulated and 177 genes differentially downregulated in the *E. faecalis* infection, as compared to the heat-killed *E. coli* control (Figure 3.6), revealing a formidable and rapid host response. In examining the genes comprising the *E. faecalis* infection gene signature (Table 3.1), I found that the top upregulated genes included *fmo-2* (flavin-containing monooxygenase), *acs-2* (fatty acid CoA synthetase), *clec-60* (C-type lectin), *mpk-2* (MAP kinase), *sodh-1* (sorbitol dehydrogenase), and *far-7* (fatty acid/retinol binding protein). The majority of the *E. faecalis*-activated genes, however, were not annotated with a known function, and many were considered "hypothetical proteins."

To gain a better understanding of the potential roles of these genes and to identify gene families that are enriched in the *E. faecalis* infection gene signature, I employed gene ontology (GO) term

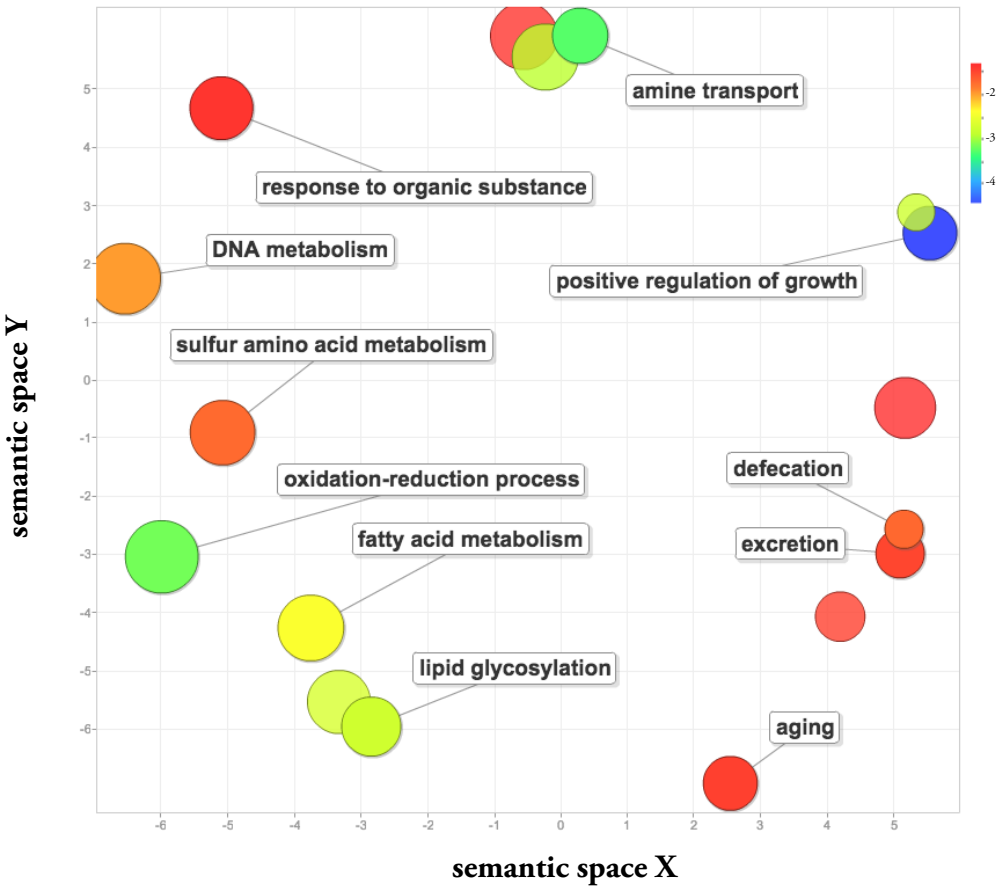


Figure 3.7: Functional classification summary of the *E. faecalis* infection gene signature. Enriched biological processes in the *E. faecalis* infection gene signature. Similar functional categories of GO terms were clustered together in two-dimensional space, using the tool REViGO. REViGO uses a simple clustering algorithm to represent GO data and represents the semantic similarity of the data on the x- and y-axes. Bubble color indicates p-value derived from DAVID and bubble size is proportional to the frequency of GO terms in the GO annotation database. Colors corresponding to $\log_{10}$ p-value are provided in the legend.

Table 3.1: *C. elegans* genes induced 4-fold or higher after 8 hours infection with *E. faecalis*, with $p < 0.05$.

Affymetrix ID	Public name	Description	Fold Change
191759_at	fmo-2	Flavin-containing MonoOxygenase family	81.107
192195_at	acs-2	fatty Acid CoA Synthetase family	36.071
192509_at	clec-60	C-type LECtin	28.541
186971_at	C23G10.11	hypothetical protein	13.539
188496_s_at		hypothetical protein, permease	12.91
182904_at	W03D2.6	hypothetical protein	11.958
189299_at		hypothetical protein, phospholipase	11.042
184993_at	H02F09.3	hypothetical protein	8.985
190545_at	mpk-2	MAP Kinase	8.742
175317_s_at	E02D9.1	hypothetical protein	8.147
190978_at	sodh-1	SOrbitol DeHydrogenase family	7.577
183652_at	far-7	Fatty Acid/Retinol binding protein	7.542
184771_at	H02F09.3	hypothetical protein, partially confirmed	7.178
184624_s_at	C25H3.10	hypothetical protein	6.862
189584_s_at	F41E6.5	hypothetical protein	6.599
187598_at	C35C5.8	hypothetical protein	6.428
172403_x_at	math-27	MATH (meprin-associated Traf homology) domain containing	6.05
173919_s_at	ttr-44	TransThyretin-Related family domain	5.848
181414_at	F41C3.1	hypothetical protein, partially confirmed	5.341
184396_at	C46H11.2	hypothetical protein, partially confirmed	5.316
172481_x_at	math-3	MATH (meprin-associated Traf homology) domain containing	5.284
182470_at	F09F7.6	hypothetical protein, partially confirmed	5.166
186521_at	F21C10.10	hypothetical protein	5.049
178069_s_at	ZC247.1	hypothetical protein, partially confirmed	5.038
184295_at	Y37H2A.14	hypothetical protein	4.956
189221_at	cyp-37B1	CYtochrome P450 family	4.944
192194_s_at	asm-3	Acid SphingoMyelinase	4.867
185270_at	irg-3	hypothetical protein	4.807
180913_at	aqp-1	AQuaPorin or aquaglyceroporin related	4.689
175993_at	gsto-1	Glutathione S-Transferase	4.638
182492_at	math-14	MATH (meprin-associated Traf homology) domain containing	4.528
180704_at	F56C3.9	hypothetical protein	4.46
191527_s_at	C07A9.8	hypothetical protein	4.388
185280_at	H43E16.1	hypothetical protein	4.323
191559_s_at	F11A5.9	hypothetical protein	4.291
185067_at	C54F6.12	hypothetical protein	4.22
186182_s_at	hrg-1	Heme Responsive Gene	4.046

enrichment for the *E. faecalis*-upregulated genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID)[68]. A large percentage of the *E. faecalis* infection gene signature is enriched in genes associated with the regulation of growth, adenyl nucleotide binding, oxidation-reduction, electron carrier activity, and fatty acid metabolism (Table 3.2). This can also be appreciated in a visualization of the same GO terms using a method called REViGO (Figure 3.7), which allows for the 2D representation of related GO terms in semantic space[205].

In examining the protein domains enriched in the *E. faecalis* infection gene signature, I also noted

Table 3.2: Enrichment of gene families in the *E. faecalis* infection gene signature for upregulated genes, based on GO-terms.

Term	Description	Count	% enrichment	*p*-value
GO:0045927	positive regulation of growth	94	15.51	1.07×10^{-3}
GO:0030554	adenyl nucleotide binding	56	9.24	5.11×10^{-3}
GO:0055114	oxidation reduction	26	4.29	3.50×10^{-3}
GO:0009055	electron carrier activity	18	2.97	2.74×10^{-3}
GO:0030246	carbohydrate binding	16	2.64	3.73×10^{-3}
GO:0030259	lipid glycosylation	7	1.16	5.09×10^{-3}
GO:0006631	fatty acid metabolic process	7	1.16	7.06×10^{-3}
GO:0015837	amine transport	6	0.99	3.17×10^{-3}
GO:0031143	pseudopodium	5	0.83	2.64×10^{-6}
GO:0006865	amino acid transport	5	0.83	4.47×10^{-3}
GO:0006643	membrane lipid metabolic process	5	0.83	5.43×10^{-3}

the upregulation of detoxification genes, including cytochrome p450 genes and UDP-glucuronosyl/glucosyltransferases (Table 3.3). Furthermore, I also identified an enrichment in genes predicted to bind nucleic acids (ATP-binding helicases and D111/G-patch domain-containing proteins), as well as some acyl-CoA thioesterases, a set of auxiliary enzymes that participate in peroxisomal lipid metabolism [70,71]. The upregulation of these genes may indicate that early in *E. faecalis* infection, *C. elegans* mounts a defense response mainly characterized by xenobiotic detoxification and potentially lipid oxidation.

Table 3.3: Enrichment of protein domains in the *E. faecalis* infection gene signature among upregulated genes, based on InterPro domains.

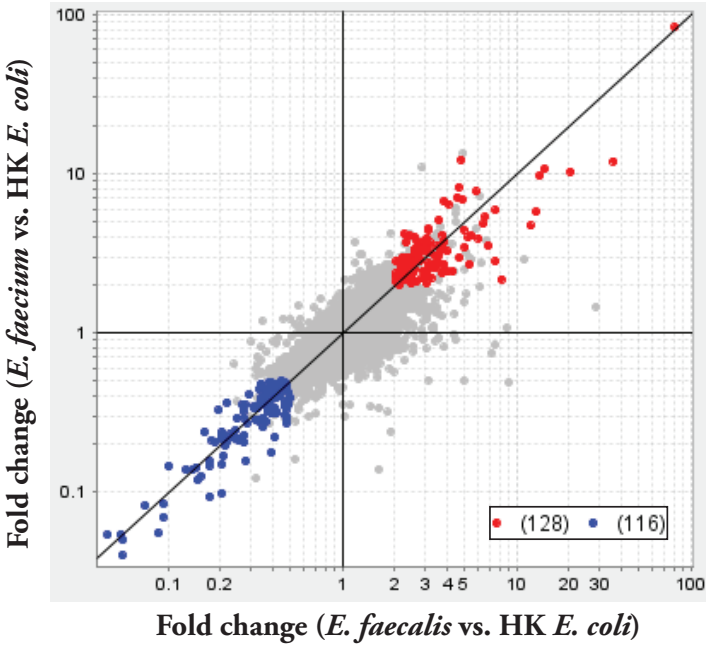
Term	Description	Count	% enrichment	*p*-value
IPR002213	UDP-glucuronosyl/UDP-glucosyltransferase	10	1.65	1.78×10^{-3}
IPR017972	Cytochrome P450, conserved site	9	1.49	3.78×10^{-3}
IPR014021	Helicase, superfamily 1 and 2, ATP-binding	8	1.32	8.66×10^{-3}
IPR000467	D111/G-patch domain	5	0.83	9.94×10^{-4}
IPR016662	Acyl-CoA thioesterase, long chain	3	0.50	6.57×10^{-3}

Alongside the *E. faecalis*-infected samples, I also profiled *C. elegans* that had been infected with

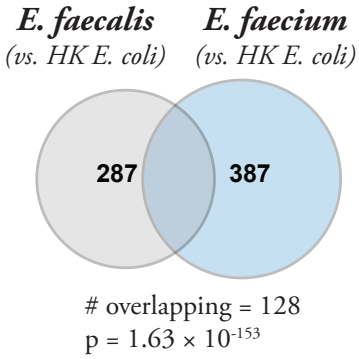
E. faecium and determined the enterococcal infection gene signature (representing the genes differentially upregulated by both *E. faecalis* and *E. faecium* infection) with respect to *E. coli*-fed *C. elegans*. I found a high degree of correlation between the *E. faecalis* and *E. faecium* infection gene signatures (Figure 3.8a). To understand the nature of the overlap between the *E. faecalis* and *E. faecium* infection gene signatures, I compared the lists of genes in each set and visualized the result using proportional Venn diagrams (Figure 3.8b). Between the 287 genes upregulated by *E. faecalis* and the 387 genes upregulated by *E. faecium*, there were 128 genes in common between the two sets, comprising a large overlap that was highly unlikely to be a result of chance ($p = 1.63 \times 10^{-153}$, hypergeometric test). Many of the genes upregulated by both *E. faecalis* and *E. faecium* were genes associated with oxidation/reduction, electron carrier activity, and iron binding (Tables 3.4, C.7); additionally, this shared enterococcal gene signature was overrepresented with flavin-containing monooxygenases and cytochrome P450s (Table C.8). The genes that were upregulated by *E. faecalis* but not *E. faecium* were enriched in genes related to amine transport and organic acid transport (Tables C.9, C.10. Conversely, the genes that were upregulated by *E. faecium* and not *E. faecalis* were enriched in ATP-binding helicases and genes relevant to DNA metabolism (Table C.11, C.12).

3.1.5 Comparison of the *E. faecalis* gene signature to other infection gene signatures

The high concordance of genes differentially expressed between *E. faecalis*- and *E. faecium*-infected worms, relative to heat-killed *E. coli* could be a consequence of the fact that *E. faecalis* and *E. faecium* are Gram-positive, whereas *E. coli* is Gram-negative. I therefore determined whether the gene signature of worms fed on the non-pathogenic, Gram-positive bacterium *B. subtilis*, also profiled in the microarray experiment, shared any similarity to that of *Enterococcus*-infected worms. Using principal component analysis, a statistical procedure that employs an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables, I observed that principal components 1 and 2, represented 85.6% of the variation observed



(a) Infection gene signatures



(b) Overlap in gene signatures

Figure 3.8: Commonalities between the *E. faecalis* and *E. faecium* infection gene signatures in *C. elegans*. (a) Fold-change by fold-change plot, comparing *E. faecalis*-infected *C. elegans* (relative to heat-killed *E. coli*-fed control) with *E. faecium*-infected *C. elegans* (relative to heat-killed *E. coli*-fed control). Highlighted in red are the genes upregulated by both *E. faecalis* and *E. faecium*; blue denotes the genes downregulated by both *E. faecalis* and *E. faecium*. In each case, differential expression was defined by fold change>2 and $p < 0.05$. (b) The set of genes upregulated in *E. faecalis* infection (287) shared a significant overlap (128) with genes upregulated in *E. faecium* infection (387). The p-value of the overlap was calculated using the hypergeometric test.

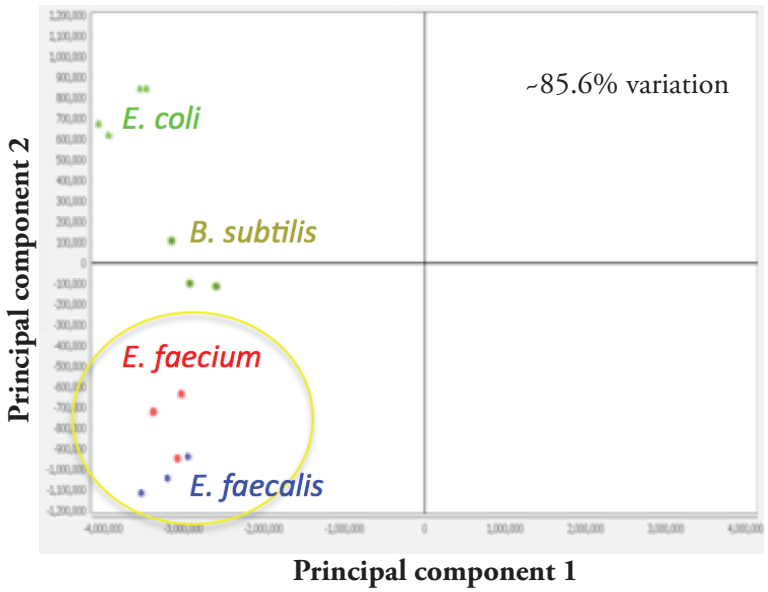


Figure 3.9: Principal component analysis of *C. elegans* fed *E. coli*, *B. subtilis*, *E. faecalis*, and *E. faecium*. A 2D projection of principal components 1 and 2, which account for 85.6% of the total variation observed in the microarray experiment comparing gene expression changes in *C. elegans* upon infection with *E. faecalis*, *E. faecium*, and *B. subtilis*, relative to heat-killed *E. coli*.

in the microarray experiments. A plot of principal components 1 and 2, showed that the *E. faecalis*- and *E. faecium*-fed samples clustered closely together, with *B. subtilis*-fed worms falling in between *Enterococcus*-infected worms and *E. coli*-fed worms (Figure 3.9).

As a complementary method to determine the relatedness of the microarray datasets, I used hierarchical clustering to compare the worms fed *E. faecalis*, *E. faecium*, *B. subtilis*, and heat-killed *E. coli*. The samples of *C. elegans* infected with *E. faecalis* clustered closely with those of *C. elegans* infected with *E. faecium*. Interestingly, some of the *E. faecium* samples clustered more closely with *E. faecalis* than with other *E. faecium* samples, and *vice versa* (Figure 3.10), which reflects just how similar the two infections are to each other in *C. elegans*. Gram-positive infection gene signatures for *Enterococcus* and *B. subtilis* also clustered together, recapitulating observations from the principal component analysis (Figure 3.9).

To investigate the degree of similarity between the *E. faecalis* infection gene signature and other pathogen infection gene signatures, I compiled and compared the datasets of previously published

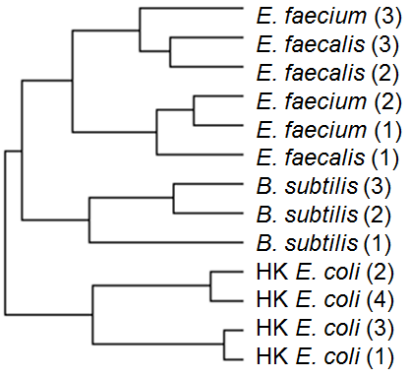


Figure 3.10: Hierarchical clustering of microarray gene expression profiles of *C. elegans* fed *E. faecalis*, *E. faecium*, *B. subtilis*, and heat-killed *E. coli*. Hierarchical clustering was performed using Pearson's correlation and the pairwise average-linkage clustering method. Each row represents one replicate.

microarray experiments that transcriptionally profiled bacterial and fungal infections in *C. elegans* using the Affymetrix *C. elegans* Genome Array (Figure 3.11). A significant overlap was detected between the genes upregulated by *E. faecalis* and *S. aureus* (Table C.3), which was enriched in genes related to oxidation/reduction and iron ion binding (Table 3.4). Additionally, there were statistically significant overlaps in genes induced by *E. faecalis* and *P. aeruginosa* (Table C.4), and even between *E. faecalis* and *Candida albicans* (Table C.5), a fungal pathogen.

I considered the possibility that some of the genes in common between these disparate pathogens were upregulated in response to a facet of the infection shared by all these infections – perhaps, something that indicated to *C. elegans* that the infection was virulent. For example, I hypothesized that some of the genes induced more highly in infection by *E. faecalis* than *E. faecium* may be indicative of the virulence of the infection. To explore this possibility, I compared the *E. faecalis* infection gene signature relative to *E. faecium* (Table C.1) to the infection gene signature of virulent *Microbacterium nematophilum*, a *C. elegans*-specific Gram-positive bacterial pathogen, relative to avirulent *M. nematophilum*, and discovered a statistically significant overlap of 20 genes (Table C.6). These genes may correspond to *C. elegans* genes induced by or specific to virulent Gram-positive infections, suggested by the enrichment of C-type lectin domains among these genes (Table 3.4).

Given that the gene expression profile of *B. subtilis*-infected worms clustered with the *Enterococ-*

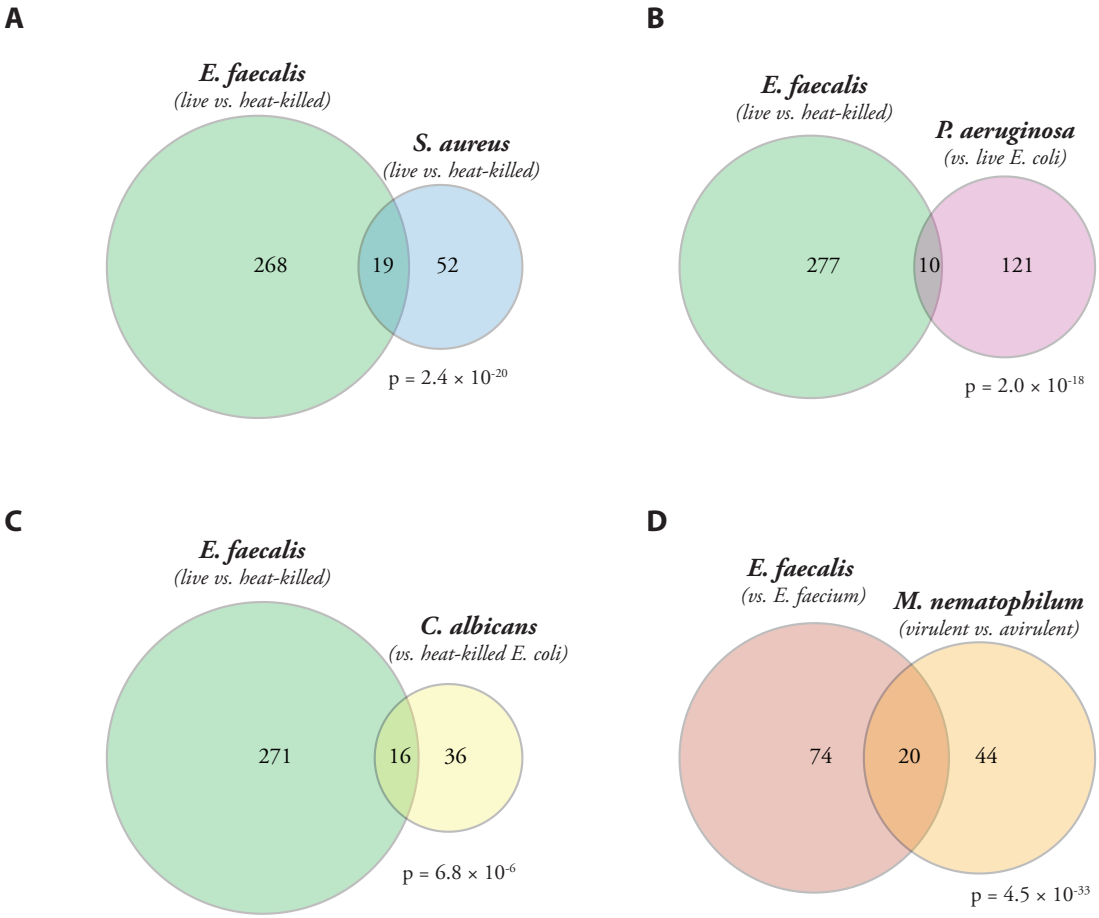


Figure 3.11: The *E. faecalis* infection gene signature comprises genes that are pathogen-specific, as well as genes that are shared among diverse pathogens. Proportional Venn diagrams comparing the *E. faecalis* infection gene signature for upregulated genes with the infection gene signature for upregulated genes for *S. aureus* (Table C.3)[79] (A), *P. aeruginosa* (Table C.4)[216] (B), and *C. albicans* (Table C.5)[167] (C). The number in the center of each circle represents the genes that are exclusively expressed by that infection gene signature. The number in the overlap of the two circles represents the genes common to both signatures. The differentially upregulated genes in *E. faecalis*, relative to *E. faecium*, were also compared to the upregulated gene signature of *M. nematophilum* infection relative to an avirulent strain of *M. nematophilum* (Table 3.4)[151] (D). The p-value of the overlap was calculated using the hypergeometric test.

Table 3.4: Enrichment of gene families and protein domains among genes differentially regulated by Gram-positive bacterial infections in *C. elegans*, based on GO-terms for gene families and InterPro protein domains.

Upregulated by both *E. faecalis* and *E. faecium*, relative to heat-killed *E. coli*

Term	Gene Family	Count	% enrichment	*p*-value
GO:0009055	electron carrier activity	11	9.40	3.80×10^{-6}
GO:0055114	oxidation reduction	12	10.26	4.59×10^{-5}
GO:0005506	iron ion binding	9	7.69	9.56×10^{-4}
GO:0004499	FMO activity	3	2.56	1.26×10^{-3}
GO:0050662	coenzyme binding	7	5.98	1.65×10^{-3}
GO:0050660	FAD binding	5	4.27	1.76×10^{-3}
GO:0050661	NADP or NADPH binding	3	2.56	4.55×10^{-3}
GO:0048037	cofactor binding	7	5.98	8.50×10^{-3}

Upregulated by both *E. faecalis* and *E. faecium*, relative to heat-killed *E. coli*

Term	InterPro Domain	Count	%	*p*-value
IPR000960	Flavin-containing monooxygenase	3	2.56	9.25×10^{-4}
IPR001128	Cytochrome P450	5	4.27	1.80×10^{-3}

Upregulated by both *E. faecalis* and *S. aureus*, relative to heat-killed *E. coli*

Term	Gene Family	Count	%	*p*-value
GO:0055114	oxidation reduction	6	33.33	2.87×10^{-5}
GO:0005506	iron ion binding	4	22.22	5.82×10^{-3}

Upregulated by both *E. faecalis* (vs. *E. faecium*) and *M. nematophilum* (vs. avirulent *M. nematophilum*)

Term	InterPro Domain	Count	%	*p*-value
IPR001304	C-type lectin	4	22.22	1.20×10^{-3}

cus-infected samples (Figure 3.10), and that the *E. faecalis* infection gene signature shared genes in common with the *S. aureus* infection gene signature (Figure 3.11), I wondered whether there was any evidence for the presence of a Gram-positive infection gene signature in *C. elegans*. I used hierarchical clustering to explore the similarities between the expression patterns of these various infection signatures in *C. elegans*, using the fold-changes for the 84 most differentially regulated genes among the 10 infection gene signatures (Figure 3.12). I restricted my analysis to these genes as their expres-

sion had the maximum variance among these samples, and showed the highest weightings in the first three principal components of the 10 gene signatures. None of these genes were differentially up-regulated by all the Gram-positive bacterial infections. The infection gene signature of *S. aureus* was most similar to the infection gene signatures of *E. faecalis* (relative to *E. faecium*) and *M. nematophilum* (virulent *vs.* avirulent), possibly suggesting that the genes induced by these pathogens are responsive to a shared feature of these virulent, Gram-positive infections, which is not represented in the enterococcal infection gene signatures, relative to heat-killed *E. coli*.

3.1.6 Immune pathways are activated by and required for defense against *E. faecalis*

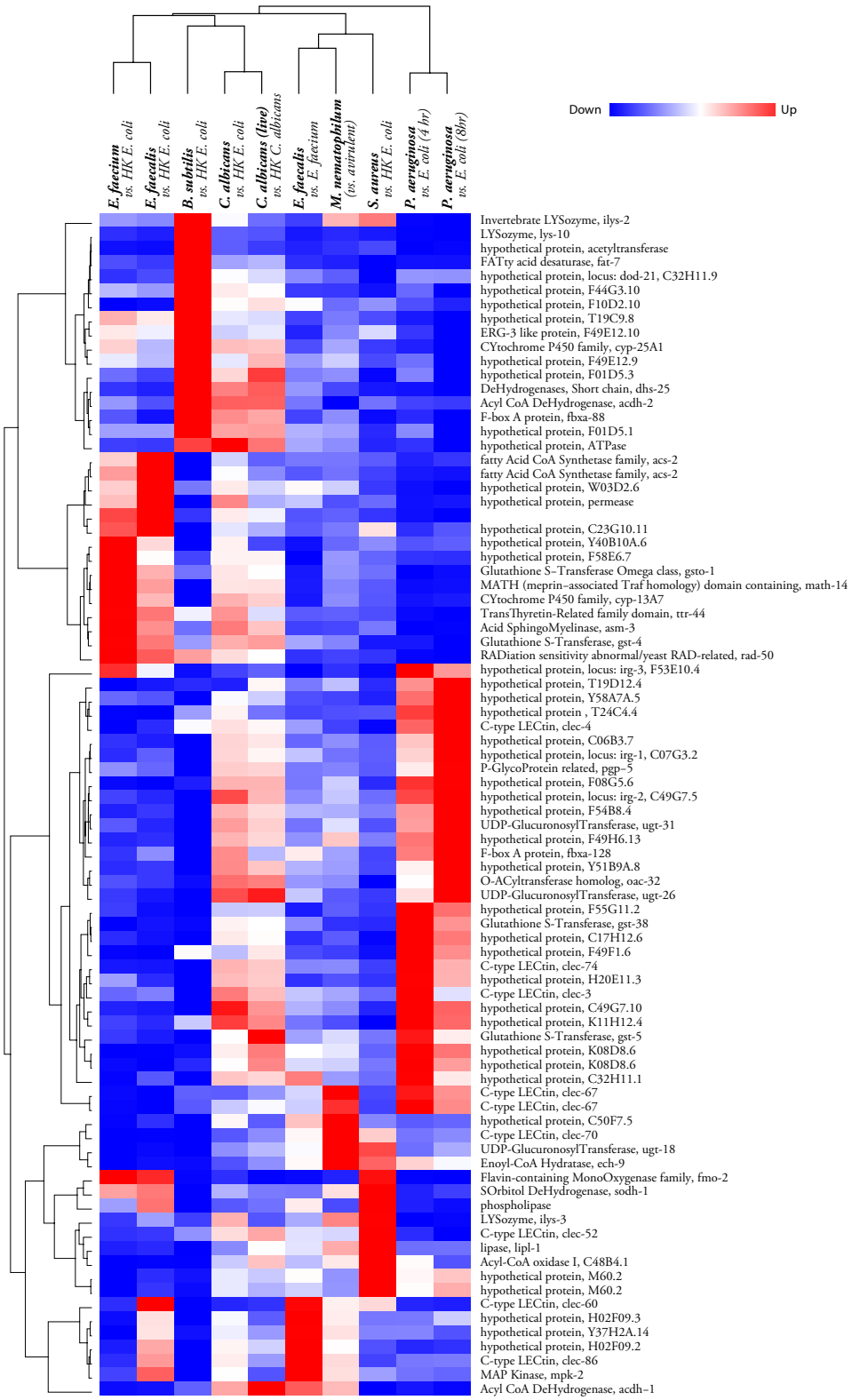
To validate the genes found to be most highly upregulated and downregulated in the microarray experiment, I took advantage of the multiplexed NanoString nCounter gene expression analysis system and designed a 72 gene "CodeSet" to verify the gene expression patterns of N2 wild-type worms on heat-killed *E. coli*, *E. faecalis*, and *E. faecium*, independently of the microarray. Despite the fact that the three different biological replicates tested in the NanoString experiment were a different set of biological replicates from those prepared for the microarray experiment, an excellent concordance was observed between the two experiments, for both *E. faecalis*- and *E. faecium*-induced genes (Figure 3.13).

Having validated the probe design for the NanoString CodeSet of putative immune genes, I next investigated whether the *Enterococcus*-induced genes were regulated by known pathways. To this end, I used the 72 gene NanoString CodeSet to profile *C. elegans* mutants deficient in *pmk-1* (the best characterized immune pathway in *C. elegans*, a p38 MAPK), *bar-1* (*C. elegans* β-catenin), *fshr-1* (a leucine-rich repeat containing G-protein coupled receptor, homologous to follicle stimulating hormone receptor), *nipi-3* (Tribbles-like kinase) and *mpk-2* (a MAP kinase predicted to function downstream of an unknown receptor tyrosine kinase) infected with *E. faecalis*, and compared the gene expression of these mutants to wild-type N2 infected with *E. faecalis* (Figure 3.14). The *mpk-2* mutant was chosen for profiling, as it is differentially upregulated during infection with *E. faecalis* but not *E. faecium*, suggesting that it may have a role in sensing the virulence of a pathogen. Additionally, *mpk-2* is highly upregulated by other virulent Gram-positive pathogens, including *M. nematophilum* and *S. aureus*.

The disruption of any of these pathways hampered the induction of at least some of the *Enterococcus*-activated genes. Interestingly, the most highly upregulated C-type lectin, *clec-60*, appears to be positively regulated by all the above immune pathways, except *mpk-2*. Most strikingly, the *fshr-1* mutant exhibited an interesting gene expression pattern, characterized by the abrogation of *clec-*

Figure 3.12 *(following page)*: Hierarchical clustering of *C. elegans* infection gene signatures. The 84 most differentially expressed genes from 10 infection gene signatures, as well as the infection gene signatures themselves, were clustered hierarchically into groups on the basis of the similarity of their expression profile, using Pearson's correlation to generate dendrograms. For each gene, the gene expression fold change is represented by color intensities (red indicates unregulated, while blue indicates downregulated).

Figure 3.12: (continued)



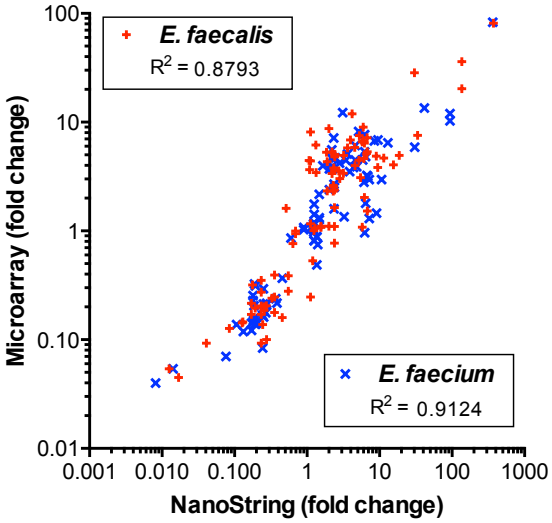


Figure 3.13: Validation of *Enterococcus*-activated genes identified in the microarray using NanoString nCounter analysis. The NanoString nCounter system was used to validate a set of 72 genes, representing the most highly upregulated and downregulated *Enterococcus*-activated genes. Correlation of microarray and nCounter data was determined by plotting the average fold difference observed in the microarray analysis (three biological replicates) versus the average fold difference for the same gene obtained by nCounter (three biological replicates, different from the microarray samples). Linear regression analysis revealed strong correlation between the microarray and NanoString datasets for both *E. faecalis* and *E. faecium*.

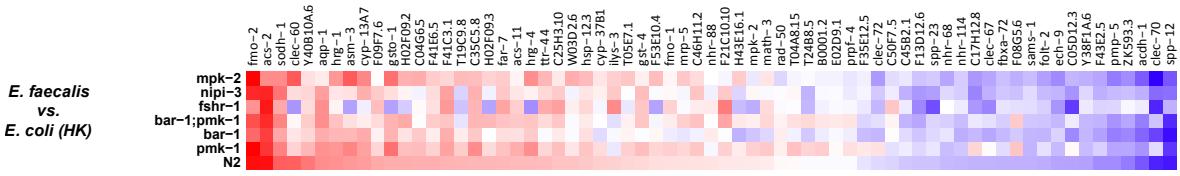


Figure 3.14: Induction of *Enterococcus*-activated genes during infection is regulated by known *C. elegans* immune pathways. Heat-map of *Enterococcus*-activated genes in wild-type N2 worms (bottom row) or immune pathway mutant worms (other horizontal rows), during infection with *E. faecalis*. Each column represents the gene, labeled at the top of the graph. The heat-map reflects data from 2-3 biological replicates for each mutant analyzed.

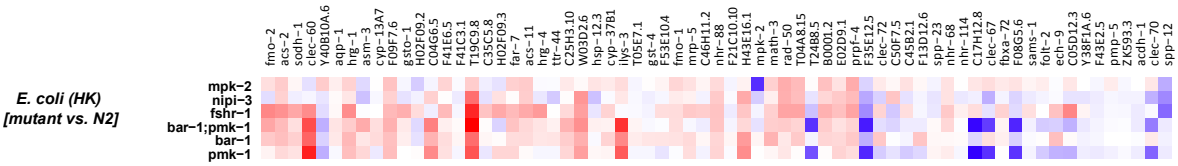


Figure 3.15: Induction of *Enterococcus*-activated genes is regulated by known *C. elegans* immune pathways, even at steady-state. Heat-map of *Enterococcus*-activated genes in wild-type N2 worms (bottom row) or immune pathway mutant worms (other horizontal rows) at steady-state (on heat-killed *E. coli*, in the absence of infection). Each column represents the gene, labeled at the top of the graph. The heat-map reflects data from 2-3 biological replicates for each mutant analyzed.

60, Y40B10A.6, hrg-1, asm-3, gsto-1, H02F09.2, hrg-4, W03D2.6, and *cyp-37B1,* among a number of other genes. This was a surprise, as *fshr-1* was identified to control a set of *P. aeruginosa* response genes, some of which are co-activated downstream of PMK-1, and was not previously thought to play an important role in the response to Gram-positive bacterial pathogens. I will return to this result and its relevance to *fshr-1* in Chapter 4 of this thesis.

I also questioned whether immune pathways regulated these *Enterococcus*-activated genes in the absence of pathogen (*i.e.*, at steady state). To explore this possibility, I compared the expression levels of the immune mutants and wild-type *C. elegans* upon feeding with heat-killed *E. coli* (Figure 3.15). Surprisingly, I found that the most highly upregulated genes in *E. faecalis* infection appear to be negatively regulated by the immune pathways (*e.g., clec-60, T19C9.8,* and *ilys-3*), as these genes were highly upregulated in the mutants at steady-state.

Given that each immune pathway tested appeared to be required for the full induction of these *Enterococcus*-activated genes in response to *E. faecalis* infection, I tested the requirement of these pathways in defense against *E. faecalis*. I compared the survival of these mutants on *E. faecalis* to that of wild-type N2 worms to probe their relative susceptibilities on *E. faecalis*. While *fshr-1* mutant worms were hypersensitive to *E. faecalis* infection, they were not nearly as compromised as the *pmk-1* mutant (Figure 3.16). The *pmk-1;fshr-1* double mutants died even more rapidly than either the *pmk-1* and *fshr-1* mutants, which may indicate that these genes act in an additive fashion in an *E. faecalis* infection, regulating independent pathways. This also appears to be the case for the BAR-1 pathway: *bar-1* mutants were hypersensitive to *E. faecalis* infection, but less so than *pmk-1* (Figure

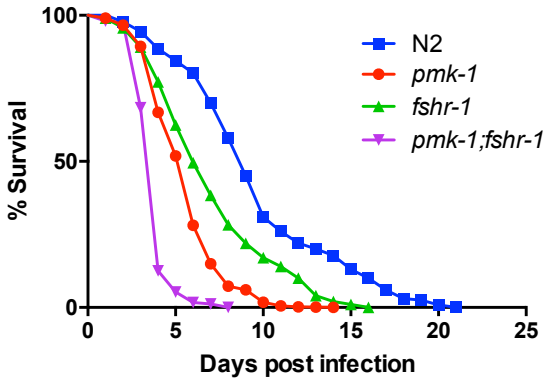


Figure 3.16: PMK-1 and FSHR-1 are required for defense against *E. faecalis*. Pathogenicity assays of N2, *pmk-1*, *fshr-1*, and *pmk-1;fshr-1* animals on *E. faecalis*. The difference between the lifespans of wild-type N2 and *fshr-1* mutant is statistically significant ($p = 2.8 \times 10^{-5}$), as are the differences in lifespan between wild-type N2 and the other mutants ($p < 1 \times 10^{-10}$). The *pmk-1* mutant was more susceptible than the *fshr-1* mutant ($p = 1.70 \times 10^{-7}$), and the *pmk-1;fshr-1* double mutant was more susceptible than the *pmk-1* mutant ($p < 1 \times 10^{-10}$).

3.17). However, the double mutant *bar-1;pmk-1* was more susceptible than either single mutant and its effect appeared to be additive, suggesting that PMK-1 and BAR-1 may act in independent pathways to mediate *C. elegans* immunity against *E. faecalis* infection.

3.1.7 Prior exposure to heat-killed *Enterococcus* protects *C. elegans* during live *E. faecalis* infection

Given the similarities between the *C. elegans* host response to *E. faecalis* and *E. faecium* infection, I wondered whether this response was driven by the perception of canonical MAMPs, and whether *C. elegans* may be recognizing structural motifs conserved between the two enterococcal species – perhaps enterococcal peptidoglycan, or some characteristic of the enterococcal cell wall – that allows *C. elegans* to perceive and then mount a defense response to the pathogen.

To test this hypothesis, I used the 72-gene NanoString CodeSet to compare the gene expression profile of N2 wild-type *C. elegans* fed heat-killed *E. faecalis* for 8 hours to that of N2 wild-type *C. elegans* fed live *E. faecalis*. Surprisingly, I found the gene expression profiles of these two samples to be extremely similar (Figure 3.18, top panel, bottom two rows). I then assessed whether the induction

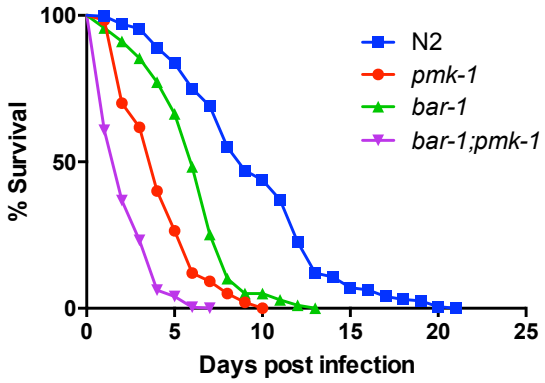


Figure 3.17: PMK-1 and BAR-1 are required for defense against *E. faecalis*. Pathogenicity assays of N2, *pmk-1*, *bar-1*, and *pmk-1;bar-1* animals on *E. faecalis*. The difference in lifespans between wild-type N2 and *bar-1* mutant is $p = 2.4 \times 10^{-5}$; the differences in lifespans between wild-type N2 and the other mutants are $p < 1 \times 10^{-10}$. The *pmk-1* mutant is more susceptible than the *bar-1* mutant ($p < 1 \times 10^{-10}$), and the *pmk-1;bar-1* double mutant is more susceptible than the *pmk-1* mutant ($p < 1 \times 10^{-10}$).

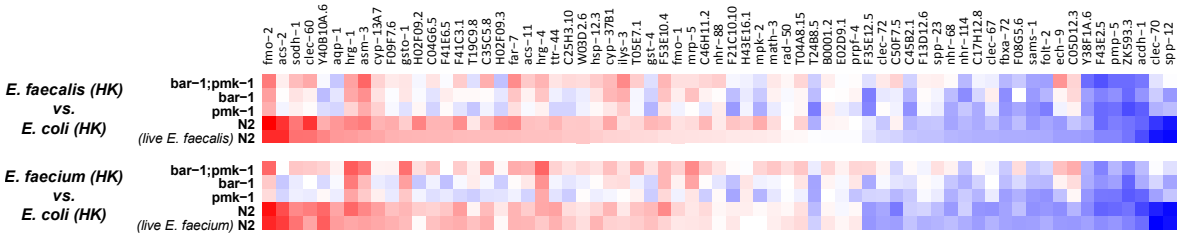


Figure 3.18: Both heat-killed and live *Enterococcus* induce the same genes to a similar magnitude. Heat-map of *Enterococcus*-activated genes in N2 wild-type or immune pathway mutant worms, after 8 hours of exposure to heat-killed *E. faecalis*. The top panel represents worms that have been treated with heat-killed *E. faecalis*; the bottom panel represents worms that have been treated with heat-killed *E. faecium*. For comparison, induction in N2 wild-type worms by live *E. faecalis* or *E. faecium* is shown in the bottom row of each panel. The heat-map reflects data from 2-3 biological replicates for mutant analyzed.

of these *Enterococcus*-activated genes by heat-killed *E. faecalis* was dependent upon the known immune signaling pathways previously identified to regulate the induction of these genes on live *E. faecalis*. Indeed, this appeared to be the case (Figure 3.18, top panel): mutants deficient in PMK-1, BAR-1, or both pathways were compromised in the induction of these *E. faecalis*-activated genes.

Given this finding, I next tested whether heat-killed *E. faecium* could also elicit the same *C. elegans* transcriptional response as live *E. faecium*, and if so, whether this response was also dependent upon previously identified immune pathways. Using expression profiling of wild-type N2 worms along

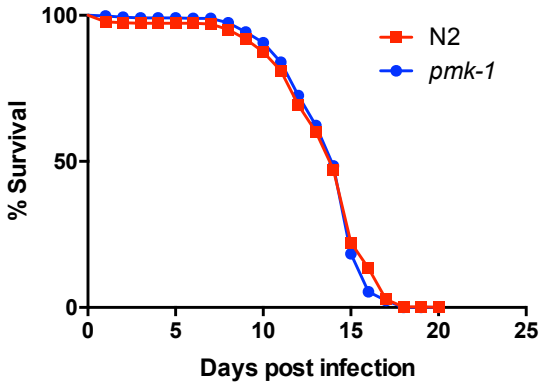


Figure 3.19: Survival of N2 and *pmk-1* worms on heat-killed *E. faecalis*. Lifespan of N2 and *pmk-1* worms sterilized with *cdc-25.1* RNAi on heat-killed *E. faecalis*. Heat-killed *E. faecalis* does not affect the lifespan of either N2 or *pmk-1* mutants. The difference in lifespan between the wild-type N2 and *pmk-1* mutant is not statistically significant ($p = 0.30$).

with the *pmk-1*, *bar-1*, or *pmk-1;bar-1* double mutant, it was noted that after 8 hours, heat-killed *E. faecium* was also able to induce the same response that live *E. faecium* did (Figure 3.18, bottom two rows), and that removal of either BAR-1, PMK-1, or both pathways, abrogated the induction of these *Enterococcus*-activated genes by heat-killed *E. faecium*. Together, these data confirm that *C. elegans* indeed responds transcriptionally to the heat-killed and live *Enterococcus* samples in a very similar manner, and that full induction of these *Enterococcus*-activated genes are dependent upon known immune pathways for *E. faecalis* and *E. faecium*, heat-killed or live.These results also imply that the perception of and response to *Enterococcus* is mediated by heat-stable moieties or components of *E. faecalis* and *E. faecium*.

Importantly, heat-killed *E. faecalis* did not kill either wild-type N2 or *pmk-1* mutant worms (Figure 3.19); additionally heat-killed *E. faecalis* does not alter defecation physiology (Figure 3.4). It will be useful in the future to determine whether heat-killed *E. faecium* is also not pathogenic to either wild-type N2 or *pmk-1* mutant worms.

Because heat-killed *E. faecalis* is not pathogenic but induces the same transcriptional response that live *E. faecalis* induces, I reasoned that an 8 hour pre-exposure of *C. elegans* worms to either heat-killed *E. faecalis* or *E. faecium* may render worms more resistant when subsequently challenged by a later

live *E. faecalis* infection. To test this hypothesis, *fer-15;fem-1* young adult *C. elegans* were exposed for 8 hours to heat-killed *E. coli*, *E. faecalis*, or *E. faecium*, or to live *E. faecalis* or *E. faecium*, after which these "pre-conditioned" worms were transferred to a lawn of live *E. faecalis* (Figure 3.20). I found that of the conditions tested, pre-conditioning of *C. elegans* with heat-killed *E. faecalis* afforded the greatest increase in resistance to *E. faecalis* (mean lifespan of 5.95 days with heat-killed *E. faecalis* treatment vs. 4.43 days of continuous live *E. faecalis* treatment, p-value = 0.04). Pre-treatment with heat-killed *E. faecium* also appeared to render *C. elegans* more resistant to some extent (mean lifespan of 5.84 days, p-value = 0.08), though this difference was not statistically significant. One explanation for heat-killed *E. faecium* and heat-killed *E. faecalis* having differential pre-conditioning abilities may be due to the different genes that they activate in *C. elegans*, not all of which are represented in the NanoString CodeSet used in these experiments. One particular point of note is that live *E. faecium* did not offer any significant resistance to subsequent *E. faecalis* infection, perhaps because *E. faecium*, while not affecting *C. elegans* lifespan, may also be slightly pathogenic to *C. elegans*, and thus pre-treatment with live *E. faecium* may do more damage to the worms than pre-treatment with heat-killed *E. faecium*. Together, these data demonstrate that *C. elegans* can be primed by heat-killed *Enterococcus* to induce a protective defense response that renders *C. elegans* more resistant to subsequent infection with live *E. faecalis*.

3.2 Discussion

Enterococcus is one of the most common nosocomial pathogens, with the majority of human enterococcal infections being caused by *E. faecalis*. As such, understanding the relationship between *E. faecalis* virulence mechanisms and the host response will help generate an integrated view of enterococcal pathogenesis. To gain further insight into these host-pathogen interactions, I used the *E. faecalis* infection model of *C. elegans*, which had been previously shown to cause intestinal distention, followed by host lethality.

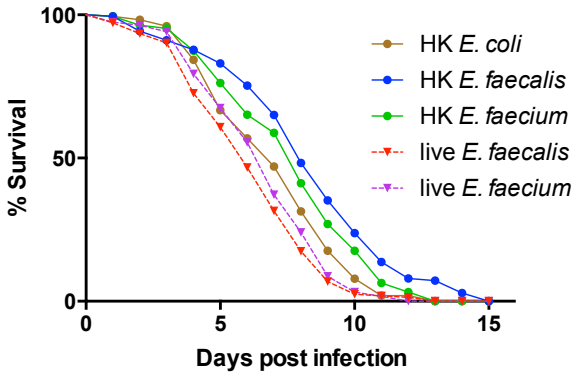


Figure 3.20: Pre-exposure to heat-killed *Enterococcus* protects *C. elegans* against later *E. faecalis* infection. Pathogenicity assay of *fer-15;fem-1* worms exposed to 8 hours of heat-killed bacteria (*E. coli*, *E. faecalis*, or *E. faecium*) or live bacteria (*E. faecalis* or *E. faecium*). There is a statistically significant difference in susceptibility to *E. faecalis* infection between *C. elegans* pre-treated with either heat-killed *E. faecalis* ($p = 2.4 \times 10^{-6}$) or heat-killed *E. faecium* ($p = 1 \times 10^{-4}$), but not heat-killed *E. coli* ($p = 0.072$) or live *E. faecium* ($p = 0.34$). In addition, the lifespan prolongation conferred by pre-treatment with heat-killed *E. faecalis* as compared to heat-killed *E. coli* was of borderline statistical significance ($p = 0.04$); the difference between the lifespans of worms pre-treated with heat-killed *E. faecium* versus heat-killed *E. coli* was not statistically significant ($p = 0.08$).

3.2.1 *E. faecalis* virulence in *C. elegans*

Ultrastructural imaging was used as a way to explore the host cellular processes disrupted during *E. faecalis* infection and showed that although *E. faecalis* kills *C. elegans*, it appears to do so insidiously, as no obvious host damage or morphological changes could be observed by electron microscopy, other than some dehiscence of the terminal web from the luminal membrane, even late in infection. With this, one would suspect that that the virulence mechanisms by which *E. faecalis* kills *C. elegans* are substantially different from that of previously described microbial infections in *C. elegans*, namely *P. aeruginosa* and *S. aureus*. While both pathogens cause gross intestinal distention in the worm, microscopically, *P. aeruginosa* infection was associated with accumulation of extracellular material, intracellular invasion, outer membrane vesicles, and abnormal autophagy, and *S. aureus* infection was associated with anal deformation, enterocyte effacement, and cell lysis. This correlated with the finding that the three pathogens activate different immune pathways in *C. elegans*.

Prior studies on host-pathogen interactions between *Enterococcus* and *C. elegans* implicated a role for many different enterococcal virulence factors in pathogenesis. The Fsr quorum sensing regulon

had been implicated in virulence, as the *E. faecalis* fsrB mutant had been shown to be attenuated in virulence in the *C. elegans* infection model. A number of toxic mediators produced by *E. faecalis* have also been thought to contribute to pathogenesis in *C. elegans*: *E. faecalis* mutants in the extracellular serine protease SprE, the secreted lytic toxin cytolysin, and the pheromone-responsive aggregation substance protein, all display attenuated virulence in the infection of *C. elegans*. The results with non-hemolytic and hyper-hemolytic strains of *E. faecalis* demonstrate that enterococcal cytolysin may have a target in *C. elegans*, as overexpression of cytolysin enhanced *E. faecalis* killing of *C. elegans*, whereas the absence of hemolytic activity slightly decreased killing. Future work, through biochemical or genetic means, will be necessary to identify the endogenous host target of enterococcal cytolysin.

It is important to note that no mutant or strain of *E. faecalis* has been identified that is completely non-pathogenic in the *C. elegans* infection model. Trivial explanations for this include the possibility that this is an indication of the difficulties of genetic manipulation of Gram-positive bacteria, especially the *Enterococci*, and that further testing will be required to identify non-pathogenic *E. faecalis* strains and mutants. Alternatively, it may also suggest that *E. faecalis* coordinates a large number of independently-acting virulence factors to mediate pathogenicity in *C. elegans* or that enterococcal virulence mechanisms at play in the *C. elegans* infection model are substantially different from what has already been explored in mammalian models.

The latter explanation is particularly interesting, supported by comparison of the virulence of the oral *E. faecalis* isolate OG1RF, a strain devoid of many pathogenicity-related genes in mammals and considered rather avirulent in humans, to V583, a vancomycin-resistant, virulent *E. faecalis* strain, of which 25% of the genome contains mobile elements, including a pathogenicity-associated island. In the *C. elegans* infection model, these two strains have comparable virulence, even though OG1RF is believed to lack many genes thought to be required for virulence in humans. One explanation is that the genes required for virulence in humans are not necessary to render *E. faecalis* pathogenic in *C. elegans*, and that factors and pathways fundamental to *E. faecalis* biology and inherent to the core

E. faecalis genome are what are truly important in *E. faecalis* pathogenicity of *C. elegans*. In analogous bacterial infection models of *C. elegans*, such as *P. aeruginosa*, there are a number of mutants (*e.g.*, *gacA* mutants; *gacA* encodes a highly conserved response regulator in Gram-negative bacteria that is required for the production of exoenzymes and secondary metabolites in *P. aeruginosa*) and naturally-occurring strains (*e.g.*, PAK) that display attenuated virulence. In the *E. faecalis* infection model of *C. elegans*, however, no such *gacA*- or PAK-equivalent *E. faecalis* strain has been identified.

3.2.2 Does *E. faecalis* cause "paralytic ileus" of the nematode?

Because *E. faecalis* had been shown to distend the *C. elegans* intestine, packing the gut to its maximum capacity of 10^6 bacteria per worm in less than 24 hours, I explored whether infection with *E. faecalis* impaired the usually stable defecation rhythm in *C. elegans*, as this may be one contributing factor to bacterial accumulation in the intestine and a possible cause of lethality. I wondered whether the observed differences in defective defecation in *C. elegans* were a cause or consequence of killing. One might expect that if a decrease in defecation is a major cause of death, then *pmk-1* mutants, which are hypersusceptible to *E. faecalis* killing, should also show increased defecation times; alternatively, if defecation is simply a consequence of the infection, one may or may not see lengthened defecation cycle lengths in the *pmk-1* mutant. The data demonstrating that *pmk-1* mutants infected with *E. faecium* exhibit greater defecation defects than wild-type N2 worms implies a correlation between a successful infection and changes in defecation. To test whether the impairment of defecation *per se* affects pathogen susceptibility, it will be worth testing a panel of defecation mutants for their sensitivities to infection with *Enterococcus*.

It was extremely intriguing that *E. faecalis* induces constipation in *C. elegans*, as the natural pathogen *M. nematophilum* is also known to cause this; thus, constipation may be a natural, physiological response of *C. elegans* following infection with particular virulent bacteria. Furthermore, *M. nematophilum* establishes a specific rectal infection by adhering firmly to the *C. elegans* cuticle, which, in turn, causes a localized swelling response in the host. This "deformed anal region" phenotype was

recognized to be the result of a protective activation of the MPK-1 ERK MAPK cascade in the region[148]. It will be worth looking closely by microscopy to see whether a similar "tail swelling" can be observed in *E. faecalis*-infected worms and determining whether MPK-1 is involved in *C. elegans* immunity against *E. faecalis*.

The *C. elegans* defecation motor program has been well worked out from a physiological as well as genetic standpoint. Mutants isolated for defective defecation phenotypes are also severely constipated, slow to mature, and are of small size – all of which suggest that these worms are not receiving adequate nutrition, despite eating[5]. Furthermore, many of the mutants that exhibited altered enteric muscle contraction also showed altered egg-laying muscle contraction phenotypes[58,59]; as a result, these defecation-defective mutants were also unable to lay eggs, even in response to excitatory transmitters thought to act directly on the egg-laying muscle. These two phenotypes – slow maturation and defective egg-laying – piqued my attention, as *E. faecalis*-infected worms are also slow to mature, scrawny and skinny, and defective in egg-laying. This last point, in particular, had initially complicated the scoring of *E. faecalis* pathogenicity assays: it was observed that *E. faecalis*-infected *C. elegans* were unable to release their eggs, to the point where the eggs matured inside the worms, hatched, and ultimately killed the hermaphrodite mother worm – a phenomenon called "bagging." To obviate this confounding factor in our pathogenicity assays, worms have to be rendered sterile, either through a temperature-sensitive genetic mutation or through feeding with a sterilizing RNAi clone before the assay. It is intriguing to speculate that the presence of a functional defect in the defecation motor program may explain other physiological effects of the *E. faecalis* infection of *C. elegans* that have been previously observed.

The physiological phenomenon of defecation impairment in *C. elegans* infected with *E. faecalis* is reminiscent of a clinical condition in humans called paralytic ileus, characterized by the obstruction of the intestine due to paralysis of the intestinal muscles[111,113]. In patients with ileus, inactivity of the intestinal muscles can prevent the passage of food, leading to a blockage of the intestine, as well as constipation and abdominal distention. Ileus can be caused by a number of factors, but it

has been well documented that noxious stimulation from the lumen, by either microbes or chemicals, can alter intestinal motility. Notably, paralytic ileus can occur even in the absence of any major structural abnormality in the intestine, a finding that can be applied to our study, as I have been unable to observe obvious structural damage of the host in *E. faecalis*-infected *C. elegans*. In studies in mammalian models, it remains to be determined how infection or injury initiated in the gut alters function or structure in the deeper neuromuscular layers, though there are already indications that inflammation can change sensory-motor functions in the gastrointestinal tract, initiating a violent response in an attempt to eliminate the noxious agent from the intestinal lumen[95]. Nevertheless, there are growing indications of a link between the host immune response and its ability to modulate gastrointestinal motility.

Is the phenomenon observed in this study truly an "ileus" of the worm? Ileus implies the cessation of peristalsis, of which it turns out *C. elegans* is capable, though in a different context than from that of mammals. As *C. elegans* feeds on bacteria, a neuromuscular pump in the pharynx captures food particles and transports it back to the intestine using two motions, pumping and isthmus peristalsis[6, 200]. Pumping is the better understood of the two actions – a cycle of contraction and relaxation that sucks in a slurry of suspended particles in liquid and then expels the liquid to trap only the particles. Isthmus peristalsis, however, is a posterior-moving peristaltic contraction of the muscles in the posterior half of the isthmus, which carries food accumulated in the anterior of the isthmus back to the terminal bulb. Pumping and isthmus peristalsis must be coordinated appropriately to move the food particles into the intestine[200]. It may be worthwhile exploring whether the "ileus" in *E. faecalis*-infected worms occurs in the very anterior of the worm, *i.e.*, in the isthmus.

What exactly about the *E. faecalis* infection is causing the defecation defect in *C. elegans*? One possibility is that *E. faecalis* produces a heat-labile toxin that decreases intestinal mobility. *E. faecalis* may also indirectly induce intestinal stasis; some characteristics of the host intestinal epithelial cell may be altered in the infection process, either by the bacteria themselves or by the host defense response, perturbing neuromuscular functions. It is possible that DAMPs or host signaling molecules

produced in response to *E. faecalis* alter neuromuscular functions. An additional reason for the impairment of host defecation may be directly related to intestinal distension: *E. faecalis* may be causing distention all throughout the intestine, including the very posterior intestinal cells that causes rectal swelling. Because these cells initiate the muscle contractions of the defecation cycle, cellular damage, severe morphological perturbation or impairment in their function may result in irregular defecation. Finally, given the high degree of distention in the *C. elegans* intestine during *E. faecalis* infection, the body wall muscles themselves may be highly perturbed, and thus their function may be altered as well. As a result, distension may exacerbate ileus.

3.2.3 Similarities between the *E. faecalis* infection gene signature and other infection gene signatures

This study, for the first time, identifies the *C. elegans* genes that are differentially regulated upon infection with *E. faecalis*, *E. faecium*, and *B. subtilis*. The transcriptional response of *C. elegans* to various microbial infections has been investigated, including Gram-positive bacterial infections like *S. aureus*, which infects the intestine; another Gram-positive bacterial pathogen *M. nematophilum*, which adheres to the *C. elegans* anus and causes rectal swelling, a Gram-negative bacterial pathogen *P. aeruginosa*, and the fungal pathogen *C. albicans*, which, upon ingestion, colonizes and pierces through the intestine. The overlap in the transcriptional response to these pathogens by *C. elegans* may reflect shared elements of a defense response against each of these pathogens.

Certainly, the overlap between the *E. faecalis* infection gene signature and *S. aureus* infection gene signature is larger than that observed between *E. faecalis* and *P. aeruginosa* or *C. albicans*, suggesting that *C. elegans* may recognize and respond transcriptionally to some facets of Gram-positive bacteria. It is tempting to think that this Gram-positive related infection gene signature is directed in response to the cell wall differences between Gram-positives and Gram-negatives (MAMPs that are different between these two groups of bacteria), but further characterization of *C. elegans* infections with Gram-positive and Gram-negative pathogens will be needed before this can be concluded, as there

are countless other differences between Gram-negatives and -positives that can explain this gene signature difference equally well (*e.g.*, virulence factors used differentially by these organisms). It is even possible that worms may be somewhat blind to MAMPs: perhaps to worms, bacteria are mainly a food source and they will secrete into the lumen whatever best digests their food (*i.e.*, a special blend of antimicrobial peptides, lysozymes, and other enzymes). Thus, while the gene expression profiling analysis appears to hint at a gene expression pattern specific to virulent Gram-positive bacterial infections, this conclusion requires further investigation, as there are relatively few transcriptional profiling experiments performed for *C. elegans* infected with either Gram-positive or Gram-negative bacteria.

How can the microarray analysis inform us of how the *C. elegans* host responds early on in the infection with *E. faecalis*? While there are some difficulties in inferring the exact cellular processes that are altered during *E. faecalis* infection, one can make some predictions with educated guesses by correlating the GO terms and protein domains enriched in the *E. faecalis* infection gene signature with what has been observed from the physiology of *E. faecalis*-infected worms. Genes associated with the regulation of growth, as well as fatty acid metabolism and amino acid transport were found to be enriched in the *E. faecalis* infection gene signature. This may be explained by the observation noted in the previous section: L4 worms with defective defecation rhythms are likely malnourished and unable to derive the nutrients necessary to progress normally to the adult stage. As a result of these nutrient deficiencies, these scrawny worms develop an altered lipid and amino acid metabolism, as the intestine of *C. elegans* is the site of digestion, drug detoxification (akin to mammalian hepatocytes), and fat storage (akin to mammalian adipocytes). Furthermore, the enrichment in genes associated with amino acid transport may be a consequence of the fact that *C. elegans* obtains the amino acids it needs for cellular and organismal function by the actions of two parallel transport routes in the apical membrane of intestinal epithelial cells, acting in parallel [108,228]. Upon infection with *E. faecalis*, lipid droplets – evolutionarily-conserved organelles that act as the sites of cellular fat storage and mobilization [126] – may be rendered dysfunctional, along with amino acid transport.

The enrichment in GO terms associated with oxidation-reduction, iron ion binding, and cytochrome P450 activity is also striking. The overrepresentation in cytochrome P450 activity indicates extensive xenobiotic metabolic activity. Additionally, the oxidation-reduction signature may be derived from Phase I detoxification reactions, as one common Phase I conversion is an oxidation reaction involving the conversion of a C-H bond to a C-OH, and a second common reaction is the reduction of a chemical through "futile cycling," in which it gains a free-radical electron, which it immediately loses to oxygen[61]. The enrichment of genes encoding iron binding proteins may be related to the fact that cytochromes, as well as catalases and peroxidases, are heme-containing proteins[117]; with the increased activity and production of these proteins, iron ion binding would consequently also increase.

3.2.4 The regulation of *E. faecalis*-activated *C. elegans* immune signaling pathways

Another interesting finding of this study is the complexity of the *C. elegans* immune signaling and regulatory network. The multiplexed gene expression data demonstrates a particularly interesting facet of *C. elegans* immunity, which is that though there are many immune pathways in *C. elegans* that contribute to host defense, most of the pathways that I have identified act independently of each other. Why does *C. elegans* have several parallel pathways responsive to infection, acting to converge upon a set of defense-responsive genes? One explanation is that these pathways are all activated by different stimuli, either host- or pathogen-derived, allowing *C. elegans* an enhanced ability to integrate, as well as fine-tune the defense response to any combination of MAMPs, DAMPs, or other elicitors, with a high degree of specificity. Furthermore, layers of genetic regulation and parallel genetic pathways may act as added insurance for *C. elegans*, as other pathogens have been shown to subvert or manipulate signaling, blocking the effects of one particular pathway. Having multiple pathways that can also activate a similar set of genes can act as a safeguard against pathogens that may try to target upstream sensors or signaling modules to thwart immune and stress defense activation.

Alternatively, these pathways may appear to act in parallel in *E. faecalis* infection, but for other in-

fections, actually are not parallel. This is one limitation of epistasis anaylsis: while it is a masterful tool for genetics, allowing for the formation of a working model for further phenotypic and molecular analyses, it is not the "last word" in any biological study. Epistasis assumes a linear series of events, whereas in biology, this is often not the case, as some pathways are very likely branched or contain multiple inputs[69]. I can easily imagine this to be the case in our studies with *E. faecalis* infection: I have only taken a "snapshot" of gene expression at 8 hours post infection, and epistasis experiments for survival have only been carried out in *E. faecalis*, and not other phenotypes associated with *E. faecalis* infection. With the presence of tissue specific regulators in a particular pathway, it may very well be the case that *pmk-1* and *bar-1* appear to act in parallel pathways in survival assays on *E. faecalis*, but not in other processes related to infection, such as intestinal distention, impairment of defecation, or gene induction. Furthermore, it may also be the case that if survival of infected worms were assessed using a different bacterial pathogen, PMK-1 and BAR-1 may appear to act in the same pathway. Yet another consideration is the factor of time: if the *E. faecalis*-infected *C. elegans* were profiled for their gene expression pattern at a different timepoint, one might just happen to see all of the *C. elegans* immune pathway mutants being unable to upregulate the same set of genes.

One pertinent example of the complexity and pleiotropy of genetic regulation in *C. elegans* can be seen in the example of the well-studied PMK-1 pathway. The evolutionarily conserved p38 MAP kinase cascade is an integral part of the response to a variety of stimuli. PMK-1, in addition to its role in immunity (through the activation of ATF-7)[194] and oxidative stress (through the activation of SKN-1)[76,221] also has a role in asymmetric cell fate in neuronal development[185,207]. It is now apparent that the MAPKKK components that are responsible for the stress-dependent activation of PMK-1 are different from those that mediate neuronal asymmetric development and innate immunity[39]. Moreover, the components that function downstream of PMK-1 remain to be characterized, as different environmental cues will elicit different PMK-1-dependent effectors (e.g., *E. faecalis*-infected worms and *P. aeruginosa*-infected worms will elicit different PMK-1 dependent genes). It is becoming increasingly clear that the PMK-1 cascade regulates the transcription of many different sets of immune

effectors in a pathogen-specific manner, and the set of pathogen-induced, PMK-1-dependent genes continues to be identified with each transcriptional profiling experiment. Thus, another critical finding of this study lies in the identification of a set of *Enterococcus*-regulated genes and the several immune pathways controlled by each. Importantly, the genes that have been identified to be regulated by PMK-1 are restricted to the pathogens that have been tested previously, which has mainly been *P. aeruginosa*[216].

3.2.5 Perception of *E. faecalis* by the *C. elegans* immune system

Perhaps the most surprising finding was that preconditioning *C. elegans* with heat-killed *E. faecalis* for just 8 hours is able to extend host survival after subsequent *E. faecalis* infection. This was a striking finding, as treatment with heat-killed *E. faecalis* elicited a response in *C. elegans* tantamount to that of a weak vaccine; it was a surprise that any effect, in fact, was observed, as "vaccines" are only effective in organisms with adaptive immunity. One might argue that the lifespan extension of *C. elegans* pretreated with heat-killed *E. faecalis* is only 1.5 days, and only marginally protective. However, it is critical to remember that the worm's lifespan is anywhere from 14-28 days in these pathogenicity assays, and even with such a modest shift in lifespan, 1.5 days may be just long enough to allow the worms to escape (recall the learned aversive behavior described in Chapter 1), survive, and lay more eggs, thus reproducing with a greater number of surviving progeny; for a nematode, the evolutionary advantage of being able to extend lifespan by a couple of days is self-evident.

Is it possible that *C. elegans* is capable of immunological memory? "Memory" in the immunological context is typically associated with the adaptive immune system, and thus is considered to be restricted to vertebrates. Several studies, however, suggest otherwise – blurring the line between innate and adaptive immunity. In a study using *P. aeruginosa* infection of *Drosophila*, it was demonstrated that "priming" *Drosophila* with a sublethal dose of *Streptococcus pneumoniae* was able to protect *Drosophila* from subsequent challenge of *S. pneumoniae* at an otherwise lethal dose, providing immunity that lasted the life of the fly[158]. Priming of *Drosophila* with heat-killed *S. pneumoniae* only

protected against subsequent live infections with *S. pneumoniae* and not any other bacterial strain, suggesting pathogen specificity in the host response. Furthermore, not every heat-killed bacterial strain tested was able to prime *Drosophila* against a subsequent infection of the same live bacterial strain. Later, the authors demonstrated that the priming-induced protection with *S. pneumoniae* was dependent upon the activation of the Toll pathway, which endowed phagocytes with the ability to recognize and kill bacteria more rapidly. These intriguing results demonstrate that long-lasting, pathogen-specific priming can occur in invertebrates, though these studies are not directly translatable to our enterococcal infections of *C. elegans*, as *C. elegans* has no mobile immune cells or immune-relevant Toll pathways.

One possibility, however, is that the intestinal cells of *C. elegans*, upon prior treatment with heat-killed bacteria, may enter an altered state of heightened immune sesntivity and awareness. A phenomenon analogous to this has been studied in the context of *Plasmodium berghei* infection of *Anopheles gambiae*. In this study, it was discovered that the breaching of the host mosquito midgut by *Plasmodium* ookinetes caused bacterial commensals to come into contact with the injured epithelial cells[179]. This conferred a robust, long-lived, enhanced antibacterial response that also decreased *Plasmodium* survival upon rechallenge by increasing the numbers of circulating granulocytes, as well as changing their binding properties and morphology. This was an extremely interesting finding, as it suggested that damage (breaching the gut epithelium) was critical for inducing the potent, "memory-like" antibacterial response, likely because the mosquito immune system was stimulated to enter a systemic state of enhanced immune surveillance.

In a similar vein, it may be possible that pre-treatment of *C. elegans* with heat-killed *E. faecalis* induces PAMP- or DAMP-triggered response in the intestinal epithelium, causing the *C. elegans* immune system to also enter a state of heightened immune surveillance; because the intestinal epithelium is now poised for pathogenic attack against a subsequent *E. faecalis* challenge. It is still not understood how the *Enterococcus* infection is perceived by the *C. elegans* host immune system: the work from this thesis certainly suggests that heat-stable components in the *E. faecalis* extract induce

protection, perhaps through the recognition of conserved structural motifs specific to pathogens. However, one cannot rule out the possibility that *E. faecalis*, even when heat-killed, may perturb cellular processes in the host, as in the case of *Plasmodium* breaching the gut epithelium and injuring intestinal epithelial cells, which allows the mosquito to sense gut bacteria, in addition to the *Plasmodium*.

As one can see, there are obvious evolutionary advantages to immune memory in every organism, including prokaryotes, which themselves are at risk for infection by viruses that can lyse them or incorporate their genetic material into their genomes. Bacteria can distinguish self from non-self through the use of clustered, regularly interspaced, short palindromic repeat (CRISPR) loci, which ultimately help to thwart bacterial replication[67]. CRISPR loci, which are present in approximately 40% of sequenced bacteria, serve to confer resistance to foreign genetic elements such as plasmids and phages, forming a type of "acquired immunity" in which CRISPR spacers recognize and cut these exogenous genetic elements in a manner analogous to RNAi in eukaryotes. One can appreciate that even in prokaryotes, evolution has selected for processes that are capable of guarding against previous pathogenic infections, as bacteria need to "remember" these infections for the sake of the survival of the colony, or at the very least, until it has produced a few divisions.

In a similar vein, it would also be advantageous for any organism, even a simple metazoan like *C. elegans*, to pass on "knowledge," perhaps through molecules encoding resistance to prior encounters, to its immediate progeny. In *C. elegans*, this has been demonstrated as transgenerational epigenetic inheritance[60]. It has now been demonstrated that *C. elegans* after an episode of viral expression, "memorizes" this experience in the form of small virus-specific interfering RNA (viRNA) molecules, which protect progeny and their descendants from the virus by silencing viral gene expression[173]. These viRNAs can be transmitted through many generations in the absence of the genetic template, and surprisingly, even in the absence of a functional small RNA-generating pathway in the progeny. These results are extremely exciting, as they are the first to provide evidence for the transgenerational inheritance of an acquired trait, induced by the exposure of organisms to a spe-

cific, biologically-relevant, physiological challenge, particularly one that has a potential evolutionary relevance to an organism – viral infection. It is possible that other defense response processes, including bacterial pathogen immunity and stress resistance, may be controlled via transgenerational inheritance (through small RNAs or histone modifications), though this is something that ought to be explored in future studies.

At this point in our study, it remains unknown whether the protective response that results from priming of *C. elegans* with *E. faecalis* is dependent upon immune signaling. It is also unknown how specific this response is to *E. faecalis*: the possibility remains to be investigated that this response is *E. faecalis* specific, though if there is enough of an overlap between the genes induced by heat-killed *E. faecalis* and other pathogens, priming of *C. elegans* with *E. faecalis* may extend the lifespan of the worm upon infection with other pathogens – or even other sources of cellular stress, like oxidative stress, heavy metal stress, or heat shock. Currently, I cannot conclude whether this finding suggests a protective stress response or a true immune-driven response directed against the bacteria to damage and kill it. It is even possible that pre-treatment with heat-killed bacteria confers a survival advantage through acclimation of *C. elegans* to a metabolic change, as it is conceivable that the transition from live *E. coli* (on which the worms are propagated) to heat-killed *E. faecalis* and then to live *E. faecalis* may be a less arduous transition than from live *E. coli* to live *E. faecalis*. In the biology of host-pathogen interactions using *C. elegans*, it is often unclear where metabolism, immunity and stress resistance meet, and where they end. The three can often be intertwined because of the complexities of the *C. elegans* system, where ingested bacteria are the diet, "microbiome," and potential pathogens all at once, adding to the existing complexity of host-pathogen interactions.

As soon as there is life, there is danger.
Ralph Waldo Emerson

4

Host stress response regulates *C. elegans* immune defense genes against *E. faecium* infection

Stress, in the physiological sense, refers to the biological response to a stimulus that disturbs the homeostasis of an organism and its cellular functions. In response to various environmental stressors, which can be internal or external, organisms elicit a coordinated physiological response to restore homeostasis. To restore homeostasis following the stress of a pathogen attack, organisms often have to activate immune and stress responses, which orchestrate many processes that eventually lead to repair, detoxification, and the restoration of homeostasis.

Like all animals, *C. elegans* needs to respond to a wide variety of signals and stresses, such as fluctuations in the levels of temperature, oxygen, moisture and food, in order to survive. The best characterized stressors in *C. elegans* include heat shock, oxidative stress, the presence of heavy metals, hypoxia, and osmotic stress. These stresses elicit a robust response in *C. elegans* characterized by behavioral, physiological, and biochemical changes, allowing it to adapt to these new conditions and maintain normal organismal and cellular function. Many of these cellular stresses have an effect on the physiology, gene expression, and signaling pathways of *C. elegans* similar to what has been observed for other animals, including mammals – highlighting the conservation of stress response pathways in metazoan evolution. Furthermore, some of these stresses, such as starvation[172] and viral infection[173], are able to induce changes in the worm that confer protection to subsequent generations, when the progeny are subjected to the same stress. In the case of nutrient deprivation, for example, a set of starvation-induced small RNAs are transmitted transgenerationally, providing a means for starved worms to genetically "imprint" subsequent generations.

As mentioned in Chapter 1, *C. elegans* has mechanisms that integrate signals from extracellular stresses, including pathogens, using a variety of signaling pathways that play key roles in development, immunity, and aging. Furthermore, many of these pathways are highly conserved from invertebrates to mammals; as such, understanding the relationships that exist between stress and immune response pathways, and identifying the elicitors and corresponding host receptors and downstream components of these pathways is of great interest.

To study the interplay between stress and immunity, I used the bacterial pathogen *E. faecium*, which had previously been shown to proliferate in and distend the *C. elegans* intestine, without causing lethality. I demonstrate that *C. elegans* deficient in the immune response genes *pmk-1*, *bar-1*, or *fshr-1* exhibit a dramatically shortened lifespan when infected with *E. faecium*, indicating that an active immune response is required for defense against *E. faecium*. Using whole-genome transcriptional profiling, I probed the *C. elegans* host response to *E. faecium*, identifying an enrichment in genes associated with the response to DNA damage. Additionally, I discovered that the *E. faecium* infection

signature contains a number of C-type lectins, as well as phase I and II detoxification enzymes. Multiplexed NanoString nCounter gene expression analysis was used to validate a set of genes, upregulated by both *E. faecalis* and *E. faecium*, and profile a panel of immune mutants on each pathogen. These studies revealed very similar profiles for both enterococcal infections. In contrast, when a number of stress-related gene mutants were profiled on these two pathogens, it was evident that while both enterococcal species induced the same defense response genes to a similar degree, the regulation of these genes was controlled by different stress response pathways. Specifically, during *E. faecium* infection, a subset of host effectors in *C. elegans* is regulated by the *C. elegans* stress-associated pathways SKN-1 and KGB-1, whereas in *E. faecalis* infection, the induction of the same set of host effectors is independent of SKN-1 and KGB-1. A similar pattern was observed upon profiling *C. elegans* mutants deficient in the small RNA processing pathway, which had previously been implicated in the response to oxidative stress. These findings provide evidence for underlying differences in the induction and regulation of host immune and stress responses against *E. faecalis* and *E. faecium* infection in *C. elegans*, and potentially, their respective virulence mechanisms.

4.1 Results

4.1.1 Like *E. faecalis*, *E. faecium* proliferates in and causes distention of the *C. elegans* intestine

Prior to this study, it had been demonstrated that *E. faecium* packs and distends the *C. elegans* intestine, but does not result in a persistent or lethal infection in the *C. elegans* intestine [47]. I used transmission electron microscopy to assess whether *E. faecium* infection induced any obvious perturbations of host cellular processes and observed that *E. faecium* proliferates in the *C. elegans* intestine, much like *E. faecalis* (compare Figure 3.1 to Figure 4.1, A and B). The intestinal microvilli appeared very straight and uniform at 8 hours after infection, even though there were many live bacteria surrounding them. Some of the bacteria could be seen undergoing binary fission, while other bacteria were

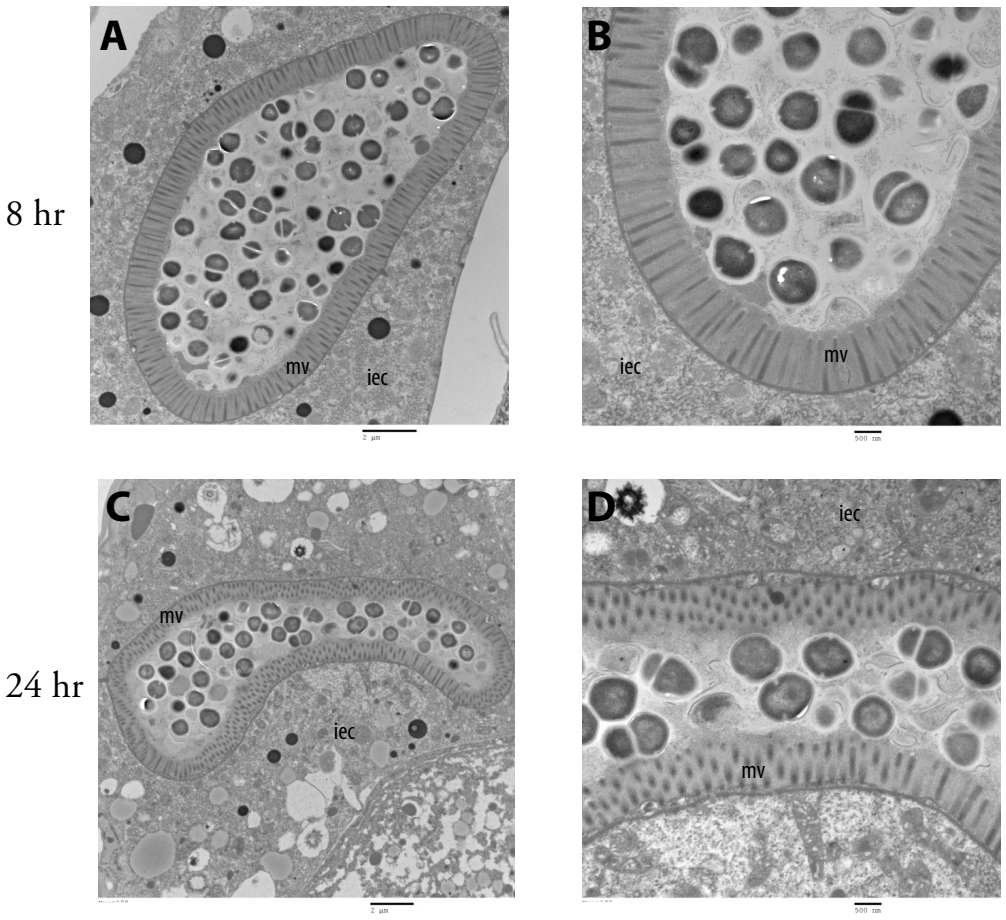


Figure 4.1: Like *E. faecalis*, *E. faecium* proliferates in and causes distention of the *C. elegans* intestine. Transmission electron micrographs of transversal midbody sections of *C. elegans* feeding on *E. faecium* E007 at 8 hours (A, B) and 24 hours (C, D). Scale bar for left images, 2 µm; scale bar for right, higher magnification images, 500 nm. The microvilli (mv) and cytoplasm (iec) of an intestinal epithelial cell are marked.

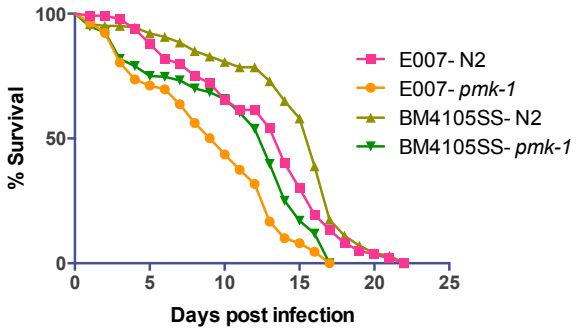


Figure 4.2: PMK-1 is required for defense against *E. faecium*. Effect of *E. faecium* infection on wild-type N2 and mutant *pmk-1* *C. elegans* using two different *E. faecium* strains, E007 and BM4105SS. *C. elegans* deficient in *pmk-1* exhibit a shortened lifespan upon infection with either *E. faecium* strain. The differences in lifespans between wild-type N2 and mutant *pmk-1* for the *E. faecium* E007 and BM4105SS strains are statistically significant ($p = 1.9 \times 10^{-7}$ and 1.6×10^{-4}, respectively).

clearly degraded or lysed, perhaps by the *C. elegans* pharyngeal grinder or by degradative enzymes and antimicrobial peptides secreted into the lumen by intestinal epithelial cells. At 24 hours post infection, there is some slight dehiscence of the terminal web from the luminal membrane, though the cause of this phenomenon is unclear. No other morphological perturbations could be observed (Figure 4.1 C, D). The bacteria at 24 hours post infection look no different from the bacteria at 8 hours post infection; some have lysed while others are still undergoing binary fission. Thus, it appears that, at least to some extent, the *C. elegans* intestine is a suitable environment for *E. faecium* to grow and proliferate.

4.1.2 An active immune response in *C. elegans* is required for defense against *E. faecium*

Given that previous studies had shown that wild-type N2 animals show no decrease in lifespan feeding on *E. faecium*, despite *E. faecium* proliferating in and packing the *C. elegans* intestine, I hypothesized that this might be a consequence of an effective immune response elicited in wild-type worms. To test whether an active immune response was required for defense against *E. faecium* strain E007, I compared the susceptibilities of wild-type N2 and mutant *pmk-1* worms to *E. faecium* infection. I initially tested the *pmk-1* mutant because the PMK-1 p38 MAPK is the most well-characterized of

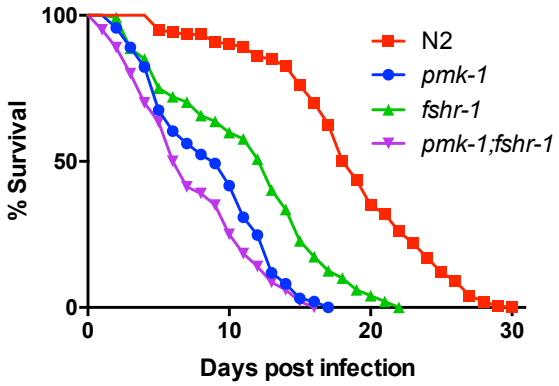


Figure 4.3: PMK-1 and FSHR-1 are required for defense against *E. faecium*. Effect of *E. faecium* infection on wild-type N2, *pmk-1*, *fshr-1*, and *pmk-1;fshr-1* animals on *E. faecium*. *C. elegans* mutants deficient in either or both pathways exhibit a shortened lifespan upon infection with *E. faecium*. The difference between N2 and each mutant tested is statistically significant ($p < 1.0 \times 10^{-10}$). The *pmk-1* mutant is more susceptible to infection than the *fshr-1* mutant ($p = 1.60 \times 10^{-6}$), and the *pmk-1;fshr-1* double mutant is more susceptible to infection than either the *fshr-1* ($p < 1.0 \times 10^{-10}$) or *pmk-1* single mutants ($p = 0.01$).

the *C. elegans* immune pathways, and because PMK-1 is required for maximal defense against all previously tested pathogens. I discovered that *pmk-1* worms exhibited a significantly shorter lifespan when feeding on the previously tested *E. faecium* strain E007 than wild-type N2 worms (Figure 4.2). This was somewhat surprising, as *pmk-1* worms have a lifespan comparable to N2 worms on *E. coli* OP50, and suggested that *E. faecium* is actually a *C. elegans* pathogen. To ensure that this finding was not specific to the particular *E. faecium* strain that was initially used, I also tested the susceptibilities of N2 and *pmk-1* to a different *E. faecium* strain, BM4105SS. This latter strain appeared to be even less pathogenic than E007, as the lifespan of wild-type N2 worms on BM4105SS is longer than N2 worms on E007, but removal of the PMK-1 pathway still caused the worms to have a shortened lifespan, demonstrating that the *pmk-1* mutant is susceptible to *E. faecium*-mediated killing by at least two *E. faecium* strains.

Given the results with *pmk-1* worms, I next evaluated the role of the FSHR-1 and BAR-1 pathways in *E. faecium* infection (Figure 4.4). Specifically, I tested a *fshr-1* and a *pmk-1;fshr-1* double mutant, as FSHR-1 was shown previously to be required for host defense against *P. aeruginosa*[163] and I found that *fshr-1* mutants are hypersusceptible to *E. faecalis* (Figure 3.16). I found that, like in the *E. faecalis*

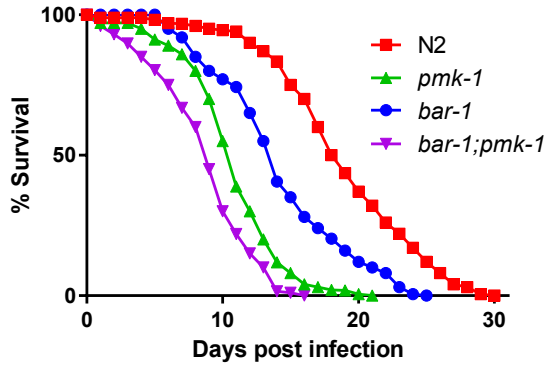


Figure 4.4: PMK-1 and BAR-1 are required for defense against *E. faecium*. Effect of *E. faecium* infection on wild-type N2, *pmk-1*, *bar-1*, and *pmk-1;bar-1* animals on *E. faecium*. *C. elegans* mutants deficient in either or both pathways exhibit a shortened lifespan upon infection with *E. faecium*. The difference between N2 and each mutant tested is statistically significant ($p < 1.0 \times 10^{-10}$). The *pmk-1* mutant is more susceptible to infection than the *bar-1* mutant ($p < 1.0 \times 10^{-10}$), and the *pmk-1;bar-1* double mutant is more susceptible to infection than either the *bar-1* ($p < 1.0 \times 10^{-10}$) or *pmk-1* single mutants ($p = 1 \times 10^{-4}$).

infection, *fshr-1* was hypersusceptible to *E. faecium* infection, but was not as susceptible as the *pmk-1* mutant, and that the double mutant was more sensitive than either of the single mutants (Figure 4.3). To test the relevance of the BAR-1 β-catenin pathway (a pathway found to be activated by and required for defense against *S. aureus* infection) in defense against *E. faecium*, I also tested single mutants in *pmk-1* and *bar-1*, as well as the *pmk-1;bar-1* double mutant and again observed that, like with the *E. faecalis* infection, the *pmk-1* mutant was more susceptible to infection than the *bar-1* mutant, and that the double mutant was more susceptible than the single mutants (Figure 4.4).

Collectively, these data demonstrate that an active immune response is required for defense against *E. faecium* in *C. elegans*. Previously, *E. faecium* had been conceptualized as a "non-pathogen" that somehow packed and proliferated in the *C. elegans* intestine, perhaps causing some morbidity. It now appears that *E. faecium* is indeed a weak pathogen and that immunocompromised worms cannot keep *E. faecium* at bay, succumbing prematurely to an *E. faecium* infection.

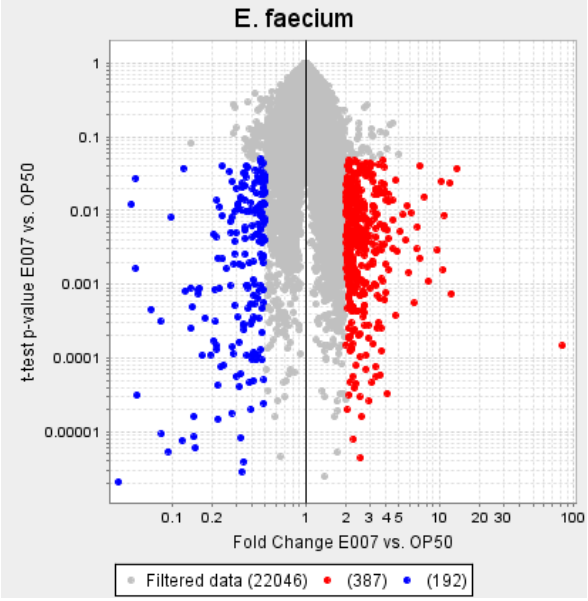


Figure 4.5: The *E. faecium* infection gene signature. Volcano plot of *C. elegans* genes that were differentially regulated in *E. faecium*-exposed versus heat-killed *E. coli*-exposed young adult animals. *E. faecium* induces a rapid response after 8 hours infection. The *C. elegans* genome array contains 22,548 sequences. Highlighted in red and blue rare the genes corresponding to the *E. faecium* infection gene signature; points colored in red represent genes upregulated by *E. faecium* (fold change > 2 and $p < 0.05$).

4.1.3 *E. faecium* infection elicits a rapid response in *C. elegans*

Given that defense against *E. faecium* requires an active host immune response, I was interested in understanding how *C. elegans* responds to *E. faecium* infection, as the ultrastructural imaging studies (Figure 4.1) did not reveal how either *E. faecium* or the *C. elegans* host were reacting during the infection process. To characterize the *C. elegans* host response to *E. faecium* infection, I used whole-genome transcriptional profiling of *C. elegans* 8 hours post infection with *E. faecium* and compared these samples to *C. elegans* worms that had been fed heat-killed *E. coli* for 8 hours. *E. faecium* induced a rapid and robust transcriptional response in *C. elegans* (Figure 4.5), with 387 upregulated genes and 192 downregulated genes with greater than 2-fold changes in gene expression ($p < 0.05$). Closer examination of the most highly upregulated genes (Table 4.1), revealed that most of the genes were annotated as hypothetical proteins, similarly to what had been found in analysis of the *E. faecalis* infection gene signature (Table 3.1). While many of the genes upregulated in *E. faecium*-infected

Table 4.1: *C. elegans* genes induced 4-fold or higher after 8 hours infection with *E. faecium*, with $p < 0.05$.

Affymetrix ID	Cosmid Name	Public name	Description	Fold Change
191759_at		fmo-2	Flavin-containing MonoOxygenase family	82.919
176851_at	Y40B10A.6	Y40B10A.6	status:Partially_confirmed	13.512
185270_at	F53E10.4	irg-3	hypothetical protein	12.262
192195_at	F28F8.2	acs-2	fatty Acid CoA Synthetase family	11.992
177544_at	F58E6.7	F58E6.7	hypothetical protein	10.88
186971_at	C23G10.11	C23G10.11	hypothetical protein	9.663
175993_at	C29E4.7	gsto-1	Glutathione S-Transferase, Omega class	8.226
173919_s_at	C56A3.2	ttr-44	TransThyretin-Related family domain	7.701
193153_s_at	T04H1.4	rad-50	RADiation sensitivity abnormal	7.14
182492_at	C16C4.4	math-14	MATH (meprin-associated Traf homology) domain containing	7.128
192194_s_at	W03G1.7	asm-3	Acid SphingoMyelinase	6.851
189815_at	T10B9.10	cyp-13A7	CYtochrome P450 family	6.713
186182_s_at	R02E12.6	hrg-1	Heme Responsive Gene	6.452
179098_at	T19C9.8	T19C9.8	hypothetical protein	6.106
190978_at	K12G11.3	sodh-1	SOrbitol DeHydrogenase family	5.883
188496_s_at			hypothetical protein, permease	5.737
190611_s_at	F22D6.5	prpf-4	vertebrate Pre-mRNA Processing Factor	5.578
189584_s_at	F41E6.5	F41E6.5	hypothetical protein	5.334
182338_at	T05E7.1	T05E7.1	hypothetical protein	5.095
187598_at	C35C5.8	C35C5.8	hypothetical protein	4.825
182904_at	W03D2.6	W03D2.6	hypothetical protein	4.694
178953_s_at	B0001.2	B0001.2	hypothetical protein	4.668
179525_at	F36H1.5	hrg-4	Heme Responsive Gene	4.488
184144_at	R193.2	R193.2	hypothetical protein, partially confirmed	4.463
189221_at	F28G4.1	cyp-37B1	CYtochrome P450 family	4.459
192673_s_at	T04A8.15	him-18	hypothetical protein	4.192
188987_at			hypothetical protein, dehydrogenase	4.176
188598_at			hypothetical protein, chitinase	4.154
176675_at	F43H9.4	F43H9.4	hypothetical protein, confirmed	4.139
190619_at	C15C8.3	C15C8.3	hypothetical protein,	4.064

worms were also upregulated in *E. faecalis*-infected worms, a few obvious differences emerged among the annotated genes. First, the C-type lectin *clec-60*, found to be upregulated 28-fold in the *E. faecalis* array, was absent from the list of the top *E. faecium*-induced *C. elegans* genes. This was a surprise, as *clec-60* is also highly upregulated by *S. aureus* and *M. nematophilum*, both virulent Gram-positive

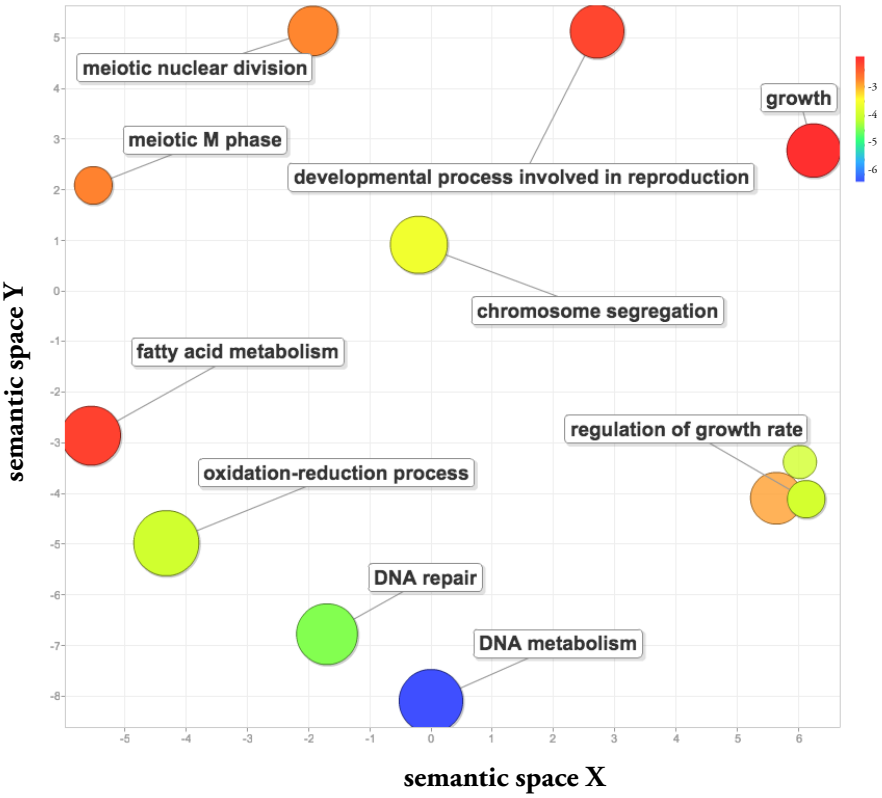


Figure 4.6: Functional classification summary of the *E. faecium* infection gene signature. Enriched biological processes in the *E. faecium* infection gene signature. Similar functional categories of GO terms were clustered together in two-dimensional space, using the tool REViGO. REViGO uses a simple clustering algorithm to represent GO data and represents the semantic similarity of the data on the x- and y-axes. Bubble color indicates p-value derived from DAVID and bubble size is proportional to the frequency of GO terms in the GO annotation database. Colors corresponding to the $\log_{10}$ p-value are provided in the legend.

bacteria. Second, the MAP kinase *mpk-2*, a putative immune-related gene, was also not differentially expressed between the *E. faecium* and *E. coli* samples. Third, the gene *irg-3* (*F53E10.4*, infection responsive gene-3) was found to be the third-most highly upregulated gene in the *E. faecium* infection gene signature. This was intriguing, as *irg-3* is also highly upregulated by *P. aeruginosa* infection.

To probe further into the function of the genes that were differentially expressed in the *E. faecium*-infected worms, I used gene ontology enrichment to identify gene families and biological processes that were overrepresented in the *E. faecium* infection gene signature. Similarly to the *E. faecalis* infection gene signature, I identified an enrichment in genes related to the regulation of growth, adenyl nucleotide binding, fatty acid metabolism, and oxidation-reduction (Figure 4.6 and Table 4.2). There

Table 4.2: Enrichment of gene families among upregulated genes in the *E. faecium* infection gene signature, based on GO-terms.

InterPro Term	Description	Count	% enrichment	*p*-value
GO:0040008	regulation of growth	95	18.77	1.26×10^{-3}
GO:0030554	adenyl nucleotide binding	59	11.66	1.15×10^{-4}
GO:0055114	oxidation reduction	30	5.93	1.16×10^{-4}
GO:0006259	DNA metabolic process	21	4.15	5.58×10^{-7}
GO:0007059	chromosome segregation	16	3.16	2.68×10^{-4}
GO:0050662	coenzyme binding	15	2.96	1.96×10^{-3}
GO:0008026	ATP-dependent helicase activity	14	2.77	6.42×10^{-7}
GO:0006974	response to DNA damage stimulus	13	2.57	2.37×10^{-5}
GO:0005783	endoplasmic reticulum	10	1.98	7.48×10^{-4}
GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	8	1.58	2.00×10^{-4}
GO:0006631	fatty acid metabolic process	7	1.38	6.60×10^{-3}

were, however, some striking differences between the *E. faecalis* and *E. faecium* infection gene signatures. Gene families that were selectively enriched in the *E. faecium* infection but not *E. faecalis* infection comprised a nexus of GO terms relating to DNA repair: DNA metabolic process, chromosome segregation, ATP-dependent helicase activity, and the response to DNA damage (Tables C.11 and C.12).

When looking further into the encoded protein domains that were enriched in the *E. faecium* infection gene signature (Table 4.3), I saw an enrichment in cytochrome P450s, glutathione S-transferases, ATP-binding helicases, and D111/G-patch domains (which are thought to bind DNA), suggesting a strong connection to xenobiotic detoxification and DNA-related biological activity.

4.1.4 Immune pathways are required for the induction of genes activated by *E. faecium*

If, as suggested by the pathogenicity assay data (Figures 4.3 and 4.4), that known immune pathways are required for defense against *E. faecium*, then known immune pathways may also be necessary

Table 4.3: Enrichment of protein domains among upregulated genes in the *E. faecium* infection gene signature, based on InterPro domains.

Term	Description	Count	% enrichment	*p*-value
IPR017972	Cytochrome P450, conserved site	13	2.57	1.57×10^{-6}
IPR014021	Helicase, superfamily 1 and 2, ATP-binding	12	2.37	4.44×10^{-6}
IPR000467	D111/G-patch domain	6	1.19	3.29×10^{-5}
IPR004046	Glutathione S-transferase, C-terminal	6	1.19	1.48×10^{-2}
IPR008139	Saposin B	5	0.99	6.37×10^{-3}
IPR018200	Peptidase C19, ubiquitin carboxyl-terminal hydrolase 2, conserved site	4	0.79	1.68×10^{-2}
IPR016662	Acyl-CoA thioesterase, long chain	3	0.59	4.88×10^{-3}
IPR000960	Flavin-containing monooxygenase FMO	3	0.59	1.61×10^{-2}

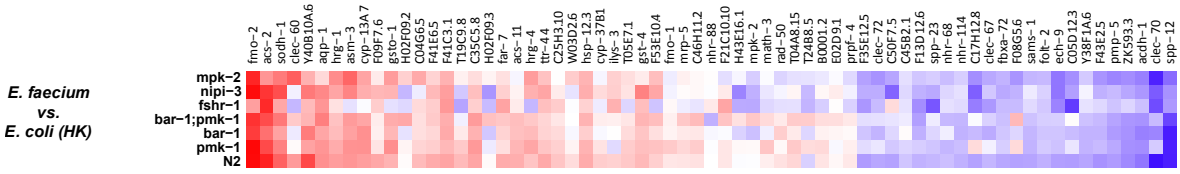


Figure 4.7: Immune pathways are required for the induction of genes activated by *E. faecium*. Heat-map of the fold-changes of *E. faecium*-activated genes in N2 wild-type or immune pathway mutant worms 8 hours after infection with *E. faecium*, relative to control heat-killed *E. coli*-fed worms. Each column represents a gene, the name of which is at the top of each column; each row represents the strain of *C. elegans* profiled.

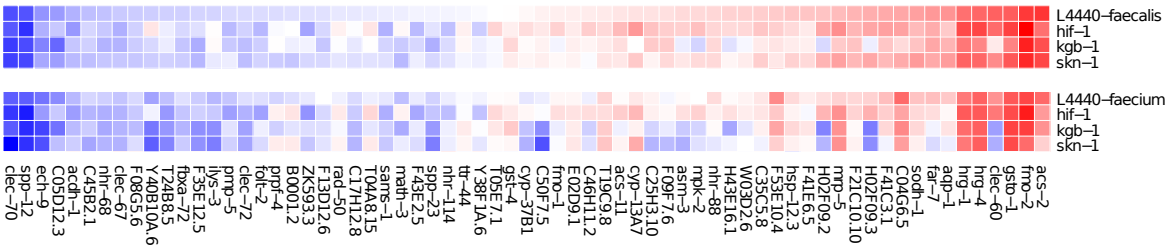


Figure 4.8: Stress response pathways are required for the induction of genes activated by *E. faecium*, but not *E. faecalis*. Heat-map of the fold-changes of *Enterococcus*-activated genes in N2 worms treated with RNAi against stress response pathway components (*hif-1*, *kgb-1*, or *skn-1*) or vector control (L4440) during infection with *E. faecalis* (top panel) or *E. faecium* (bottom panel). Expression was compared relative to heat-killed *E. coli* control. Each column represents a gene, the name of which is at the top of each column; each row represents the RNAi clone used. The heat-map reflects data from 1-2 biological replicates for each RNAi clone analyzed.

for the observed induction of the *E. faecium*-activated genes in the microarray analysis. Using the NanoString CodeSet designed for the *E. faecalis* infection studies in Chapter 3, I profiled wild-type N2 worms, along with a number of immune mutants deficient in the following genes: *pmk-1*, *bar-1*, *pmk-1;bar-1*, *fshr-1*, *nipi-3*, and *mpk-2* (Figure 4.7). I found that the gene expression profiles for these mutants on *E. faecium* were very similar to the profiles for these mutants on *E. faecalis* (Figure 3.14). No single mutant seemed to abrogate the induction of all the *E. faecium*-activated genes, though removal of any one of the pathways affected the induction of at least some of the *E. faecium*-activated genes. It also appeared that wild-type expression of some genes (*e.g.*, *clec-60*, *F09F7.6*, *T19C9.8*, *H02F09.2*, *H02F09.3*, and *T24B8.5*) depended on multiple pathways; this was also observed for these mutants when infected with *E. faecalis*.

4.1.5 *Enterococcus*-activated effectors are induced by both enterococcal species, but are regulated by different pathways

Having established that known immune pathways are required for the induction of *E. faecium*-activated genes and for *C. elegans* defense against *E. faecium*, I next investigated whether previously identified stress response pathways were also required for the induction of these *Enterococcus*-activated genes, as many phase I and II detoxification genes were found to be upregulated by *E. faecium*. To this end, I

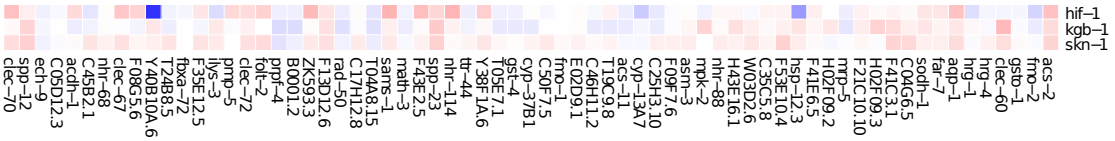
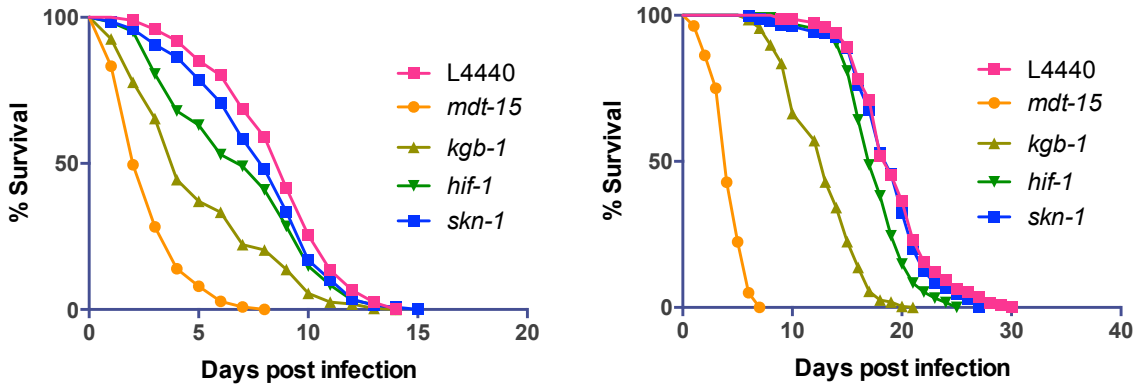


Figure 4.9: Stress response pathways are required for the induction of *Enterococcus*-activated genes at steady-state, in the absence of pathogen. Heat-map of the fold-changes of *Enterococcus*-activated genes in N2 worms treated with RNAi against stress response pathway components (*hif-1*, *kgb-1*, or *skn-1*) or vector control (L4440) fed heat-killed *E. coli*, a non-pathogenic food source). Each column represents a gene, the name of which is at the top of each column; each row represents the RNAi clone used. The heat-map reflects data from 1-2 biological replicates for each RNAi clone analyzed.

used RNAi to knock down three genes involved in stress responses: *hif-1* (hypoxia-inducible factor-1, a transcription factor responsive to hypoxia)[34,189], *kgb-1* (a JNK kinase that regulates the response to pore forming toxins and heavy metal stress)[135], and *skn-1* (a transcription factor first characterized for its role in the oxidative stress response[2] and that is now known to also exert a role in the unfolded protein response)[53]. Comparing the worms that had been treated with RNAi against stress-related genes to a vector control (L4440), I observed that the induction of a sizable subset of the *Enterococcus*-activated genes was abrogated when *kgb-1* and *skn-1* were knocked down (Figure 4.8, lower panel). Notably, upregulation of *clec-60*, *aqp-1*, *far-7*, *H02F09.2*, *H02F09.3*, *H43E16.1*, *mpk-2*, *asm-3*, *F09F7.6*, and *C25H3.10* were impaired in the absence of *kgb-1* or *skn-1*. The levels of repression of the *Enterococcus*-activated genes observed in *skn-1* and *kgb-1* suggest that these two genes modulate a very similar set of target genes, or that they act together, perhaps in the same genetic pathway. Surprisingly, this set of genes was almost identical to the genes whose induction was abrogated in the *fshr-1* mutant (Figure 3.14 and Figure 4.7). Indeed, the *fshr-1* mutant exhibited a drastically different profile from the other immune mutants, instead exhibiting an effect on gene expression similar to that of the stress mutants. This strongly suggests that *fshr-1* may play a role in the regulation of stress-induced genes, and perhaps even specifically in oxidative stress, as *kgb-1* and *skn-1* have both been implicated in the response to oxidative stress.

To determine whether the *hif-1*, *kgb-1*, and *skn-1* stress response pathways were also required for the activation of the genes activated during an *E. faecalis* infection, I repeated the RNAi knockdown experiments, this time, exposing the worms to *E. faecalis*. Unexpectedly, I found that knockdown of

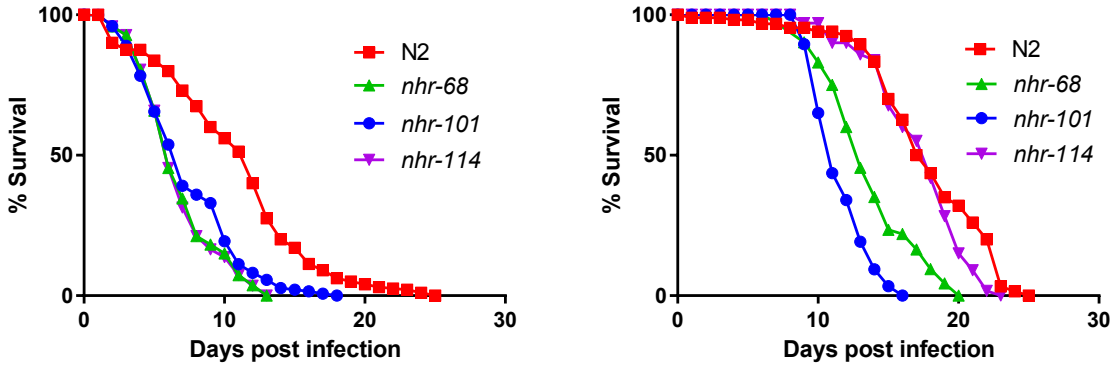


(a) *E. faecalis* **(b)** *E. faecium*

Figure 4.10: Stress response pathways are required for defense against *Enterococcus*. Effect of *E. faecium* infection on *fer-15;fem-1 C. elegans* treated with L4440 vector control, *mdt-15*, *kgb-1*, *hif-1* or *skn-1* RNAi strains. *C. elegans* mutants deficient in *kgb-1* and *mdt-15* both exhibit shortened lifespans upon infection with *E. faecalis* and *E. faecium* ($p < 1.0 \times 10^{-10}$ for each comparison). The difference in lifespans between wild-type N2 and *hif-1* is statistically significant for both enterococcal infections (*E. faecalis*: $p = 6.7 \times 10^{-3}$, *E. faecium*: $p = 1.0 \times 10^{-4}$), whereas the difference between N2 and *skn-1* is not statistically significant for either enterococcal strain (*E. faecalis*: $p = 0.22$, *E. faecium*: $p = 0.26$).

these genes barely affected the induction of the *E. faecalis*-activated genes, save for *kgb-1*, which did reduce the induction of the above-named effectors, though to much lesser extent than what had been observed in the *E. faecium* infection (Figure 4.8, upper panel). These data demonstrate that although *E. faecalis* and *E. faecium* induce the same *C. elegans* host genes, sometimes even to similar levels, the expression of these genes is regulated by different signaling pathways in these two infections. Induction of these *Enterococcus*-induced genes appears to be far more dependent upon stress response signaling pathways in the case of *E. faecium* infection than in the *E. faecalis* infection. However, both immunity and stress pathways are required for the induction of *E. faecium*-activated genes.

Next, I examined whether *hif-1*, *kgb-1*, and *skn-1* were required for the basal expression of these *Enterococcus*-activated genes, in the absence of any pathogen. Comparing these knocked-down worms to the vector control-fed worms, all exposed to heat-killed *E. coli*, I found that these stress-related genes play some role in the basal expression of the *Enterococcus*-activated genes (Figure 4.9), though not to the same degree that the previously examined immune pathways (PMK-1, BAR-1, *etc.*) reg-



(a) *E. faecalis* **(b)** *E. faecium*

Figure 4.11: Nuclear hormone receptors are required for defense against *Enterococcus*. Effect of *E. faecium* infection on wild-type N2, *nhr-68*, *nhr-101*, *nhr-114*. *C. elegans* mutants deficient in *nhr-68* and *nhr-101* exhibit a shortened lifespan upon infection with both enterococcal strains ($p < 1.0 \times 10^{-10}$ for both strains, on *E. faecalis* and *E. faecium*). *C. elegans* deficient in *nhr-114* are also immunocompromised upon infection with *Enterococcus* (*E. faecalis*: $p = 2.5 \times 10^{-7}$, *E. faecium*: $p = 6.4 \times 10^{-3}$).

ulated the steady-state levels of these genes (Figure 3.15). This may indicate that the control of these *Enterococcus*-activated genes, in the absence of any pathogen, is mediated primarily by immune-related signaling pathways and not stress-related pathways.

Given that stress response pathways are selectively required for the regulation of a subset of *Enterococcus*-activated genes in the *E. faecium* but not *E. faecalis* infection, I tested the role of these stress response pathways in protection from *E. faecium* infection to assess whether there was any difference between the susceptibilities of these mutants when infected with either enterococcal species. To test this, I RNAi-treated *C. elegans* L4 larvae and exposed them to either *E. faecalis* (Figure 4.10a) or *E. faecium*(Figure 4.10b), and found that worms deficient in *kgb-1* were hypersensitive to both enterococcal infections. While *hif-1* may play a modest role in defense against *E. faecalis* and *E. faecium*, as the difference in lifespan between wild-type and *hif-1*-deficient worms is statistically significant in both enterococcal infections, I did not observe a large or significant difference in the susceptibility of worms deficient in *skn-1*, relative to the L4440 vector control, even though *skn-1* seemed to play a larger role in the induction of the *Enterococcus*-activated genes to the *E. faecium* infection.

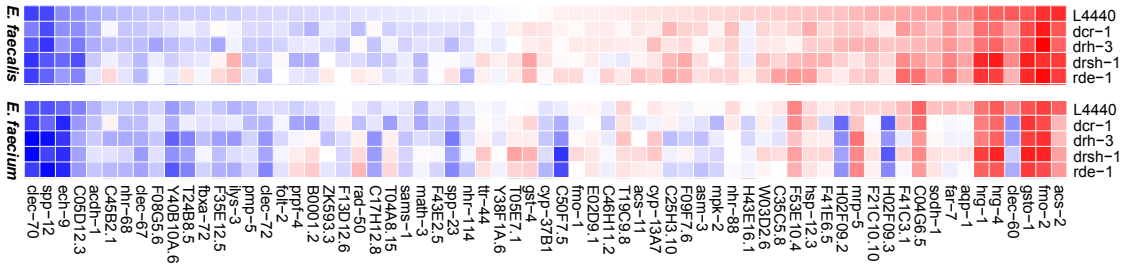


Figure 4.12: *C. elegans* small RNA pathway is required for the induction of *Enterococcus*-activated genes. Heat-map of the fold-changes of *Enterococcus*-activated genes in N2 worms treated with RNAi against *C. elegans* small RNA pathway components or vector control (L4440, top row) during infection with *E. faecalis* (top panel) or *E. faecium* (bottom panel). Expression was compared relative to heat-killed *E. coli* control. Each column represents a gene, the name of which is at the top of each column; each row represents the RNAi clone used.

MDT-15, a subunit of the conserved Mediator complex homologous mammalian MED15, has been demonstrated to activate genes in response to oxidative stress and xenobiotic treatment[54]. Additionally, MDT-15 has been shown to have a protective role against infection with *P. aeruginosa* by upregulating a number of detoxification genes downstream of PMK-1[169]. Knockdown of *mdt-15* caused enhanced susceptibility to *E. faecalis* and *E. faecium* infection, with the most severe phenotypes of the RNAi knockdowns tested, with even greater susceptibility than the *kgb-1* mutants (Figure 4.10). One would surmise that MDT-15 may play a protective role in *Enterococcus* infection by transcriptionally regulating *C. elegans* defense genes, especially detoxification-related genes, many of which are enriched in the *Enterococcus* gene signature. It is important to note, however, that the *mdt-15* mutant has a reduced lifespan when grown on the normal laboratory food source *E. coli* OP50 compared to wild-type controls[209].

Because the stress-response pathways tested were not differentially required for protection against infections with the two enterococcal species, I wondered whether other pathways related to stress might explain the transcriptional response of stress-related genes in these two infections. I focused my attention on three nuclear hormone receptors (NHRs) that were found to be differentially expressed in the *E. faecalis* and *E. faecium* arrays: *nhr-68*, *nhr-101*, and *nhr-114*. NHRs have been demonstrated to regulate gene expression in response to many cues, including developmental, environmental, and nutritional signals, and as a result, integrate numerous inputs to fine-tune the result-

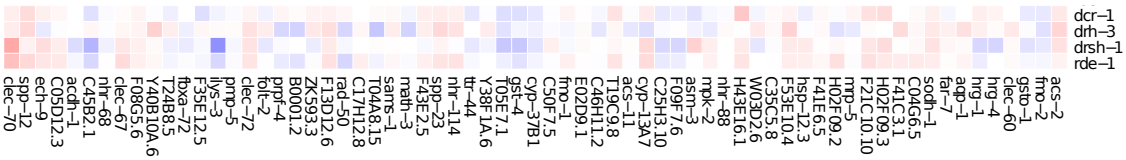
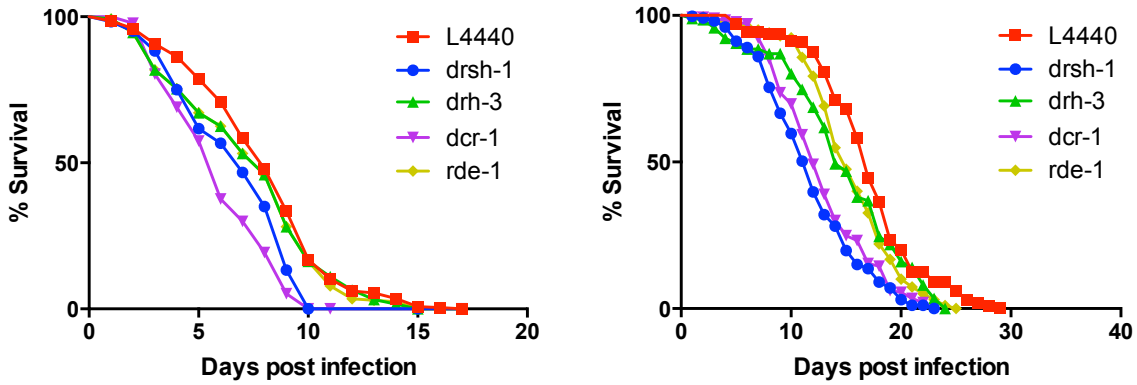


Figure 4.13: The *C. elegans* small RNA pathway is required for the induction of *Enterococcus*-activated genes at steady-state, in the absence of pathogen. Heat-map of the fold-changes of *Enterococcus*-activated genes in N2 worms treated with RNAi against small RNA pathway components or vector control (L4440) fed heat-killed *E. coli*, a non-pathogenic food source). Each column represents a gene, the name of which is at the top of each column; each row represents the RNAi clone used. The heat-map reflects data from 1-2 biological replicates for each RNAi clone analyzed.

ing transcriptional response[210]. To assess the role of these three NHRs in *C. elegans* defense against *Enterococcus*, I tested mutants deficient in each of the three NHRs for their susceptibility to *E. faecalis* and *E. faecium*. I found that all the NHR mutants tested were susceptible to both enterococcal infections, though it appears that the *nhr-114* mutant is more susceptible to *E. faecalis* than it is to *E. faecium* (Figure 4.11).

Another group of genes that were differentially upregulated in the *E. faecium* infection gene signature but which were not upregulated by *E. faecalis* consisted of small RNA processing components. Four genes, *dcr-1* (Dicer), *drh-3* (Dicer-related helicase-3), *drsh-1* (Drosha) and *rde-1* (one of the many *C. elegans* argonaute proteins) were differentially upregulated in the *E. faecium* array, but not in the *E. faecalis* array. I profiled the gene expression of *C. elegans* that had been treated with RNAi to knockdown each of these four small RNA pathway components, exposed them for 8 hours to either *E. faecalis* or *E. faecium*, and then assayed their gene expression profiles for the *Enterococcus*-activated genes represented by the NanoString CodeSet. Similarly to what was observed with worms deficient in stress response pathways, *C. elegans* deficient in components of the small RNA processing pathway infected with *E. faecalis* showed little difference in gene expression when compared with the L4440 vector control (Figure 4.12). In contrast, when the small RNA processing pathway was knocked down before infection with *E. faecium*, the induction of the same set of stress-regulated genes as in Figure 4.10 were abrogated, suggesting that the small RNA processing pathway and stress response pathways may regulate the same set of target genes in response to the same cue, or act in



(a) *E. faecalis* **(b)** *E. faecium*

Figure 4.14: Small RNA pathway components are required for *C. elegans* defense against *Enterococcus*. Effect of *Enterococcus* infection on *fer-15;fem-1 C. elegans* treated with L4440 vector control, *drsh-1*, *drh-3*, *dcr-1* or *rde-1* RNAi. *C. elegans* mutants deficient in *dcr-1* and *drsh-1* both exhibit shortened lifespans upon infection with *E. faecalis* and *E. faecium*. The difference in lifespan between L4440 and *dcr-1* (*E. faecalis*: $p = 7.3 \times 10^{-9}$, *E. faecium*: $p < 1.0 \times 10^{-10}$) and between L4440 and *drsh-1* (*E. faecalis*: $p = 1.0 \times 10^{-4}$, *E. faecium*: $p = 1.0 \times 10^{-10}$) are statistically significant. In contrast, the differences in lifespans between the L4440 vector control and the mutants *drh-3* and *rde-1* are statistically significant for the *E. faecium* infection ($p = 6.3 \times 10^{-3}$ and $p = 3.3 \times 10^{-3}$, respectively), but not for the *E. faecalis* infection ($p = 0.52$ and $p = 0.20$, respectively).

the same pathway. Indeed, mutants in *dcr-1* are compromised in the resistance to heat shock and oxidative stress[136]. These data provide additional evidence for the differential regulation of *Enterococcus*-activated genes in the *E. faecium* infection. The role of the small RNA processing pathway on the regulation of these *Enterococcus*-activated genes at steady-state, in the absence of pathogen was also examined. As I had established for the stress response pathway genes, the small RNA pathway regulated the basal expression of these *Enterococcus*-activated genes in the absence of pathogens (Figure 4.13).

To evaluate the role of the small RNA processing machinery in defense against *Enterococcus*, these genes were knocked down and then assayed for their susceptibility to *E. faecalis* and *E. faecium*. Worms deficient in *dcr-1* or *drsh-1* displayed enhanced susceptibility to *E. faecalis* and *E. faecium* infection, as were worms deficient in *drsh-1* (Figure 4.14). Interestingly, *C. elegans* deficient in *drh-3* and *rde-1* were susceptible to infection with *E. faecium*, but not *E. faecalis*, as the difference in lifespans between

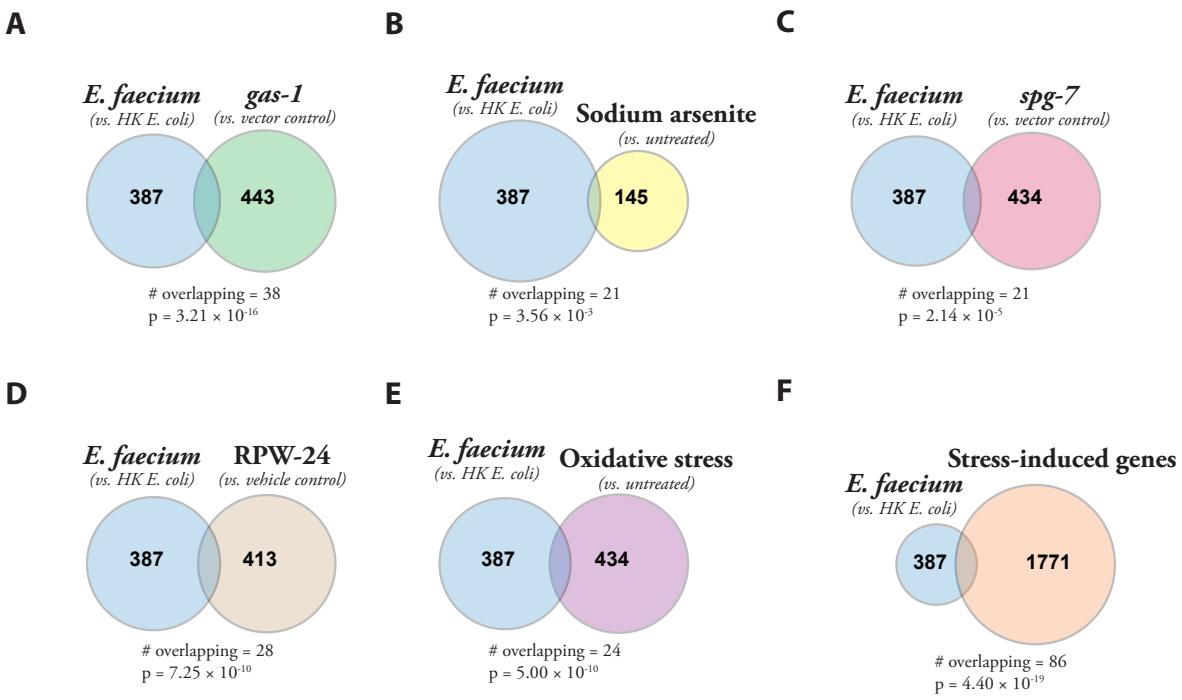


Figure 4.15: The *E. faecium* infection gene signature comprises genes that are upregulated in response to diverse cellular stresses. Proportional Venn diagrams comparing the *E. faecium* infection gene signature of upregulated genes to the gene signatures for upregulated genes of *C. elegans* deficient in *gas-1*[40] (A), treated with sodium arsenite[186] (B), treated with *spg-7* RNAi[146] (C), treated with the small molecule RPW-24[168] (D), and treated with hyperbaric oxygen (to induce oxidative stress)[155] (E). The number in the center of each circle represents the total number of genes that are upregulated by treatment with the particular indicated stress. The number in the overlap of the two circles represents the genes common to both signatures. The common enterococcal infection gene signature (genes that are upregulated by both *E. faecalis* and *E. faecium*) was also compared to the total set of genes upregulated by any of the above stress treatments (F). The heat-map reflects data from one replicate for each mutant analyzed.

the vector control and knockdowns in *E. faecalis* infection did not reach statistical significance. These results suggest that the small RNA processing pathway in *C. elegans* is required for defense against *Enterococcus*.

At this juncture in the study, I was intrigued by the differential regulation of defense genes induced by *E. faecium*. Furthermore, I wondered about the relationship between the immune and stress responses in *C. elegans*, and I speculated that there may be a shared gene signature between the *E. faecium*-activated genes and the stress response gene signatures of previously published studies. To assess this, the *E. faecium* gene signature was compared to five gene signatures, compiled from

experiments of *C. elegans* deficient in *gas-1* (a subunit of mitochondrial complex I that is required for oxidative phosphorylation, mutation of which induces mitochondrial dysfunction)[40], exposed to sodium arsenite (an inhibitor of protein translation and inducer of oxidative stress)[186], exposed to with *spg-7* RNAi (RNAi of a nuclear-encoded mitochondrial metalloprotease, which ultimately induces mitochondrial stress)[146], exposed to RPW-24 (a small molecule that, in addition to activating the PMK-1 immune pathway, also induces a potent detoxification response)[168], and exposed to hyperbaric oxygen (which induces oxidative stress)[155] (Figure 4.15, A-E). In each case, a significant overlap in the *E. faecium* infection gene signature and the stress response could be identified. In terms of the number of genes that overlapped in each comparison, 38 genes were shared between *E. faecium* and *gas-1*, 21 between *E. faecium* and sodium arsenite, 21 between *E. faecium* and *spg-7* RNAi, 28 between *E. faecium* and RPW-24, and 24 between *E. faecium* and hyperoxia.

Table 4.4: Enrichment of gene families in genes induced by *Enterococcus* and organismal stress, based on GO terms.

InterPro Term	Description	Count	% enrichment	*p*-value
GO:0055114	oxidation reduction	17	21.79	1.00×10^{-11}
GO:0005506	iron ion binding	11	14.10	1.67×10^{-6}
GO:0020037	heme binding	8	10.26	1.68×10^{-5}
GO:0007568	aging	6	7.69	6.69×10^{-3}
GO:0004364	glutathione transferase activity	3	3.85	4.27×10^{-3}

I then compared the common enterococcal signature (*i.e.*, those upregulated genes that are common to both the *E. faecalis* and *E. faecium* gene signature) to the total set of genes upregulated by any of the above stress treatments (Figure 4.15, F) and identified 49 genes shared between the two sets. To identify the gene families that were enriched in this gene set, I used gene ontology (DAVID)[68] and visualized the result using REViGO[205] (Figure 4.16 and Table 4.4). This identified several gene families relating to the oxidation-reduction process, iron ion binding, organismal aging, lipid modification, and glutathione transferase activity, suggesting that *E. faecium* and other stress responses induce changes in lipid and iron metabolism, as well as the escalation of detoxification programs.

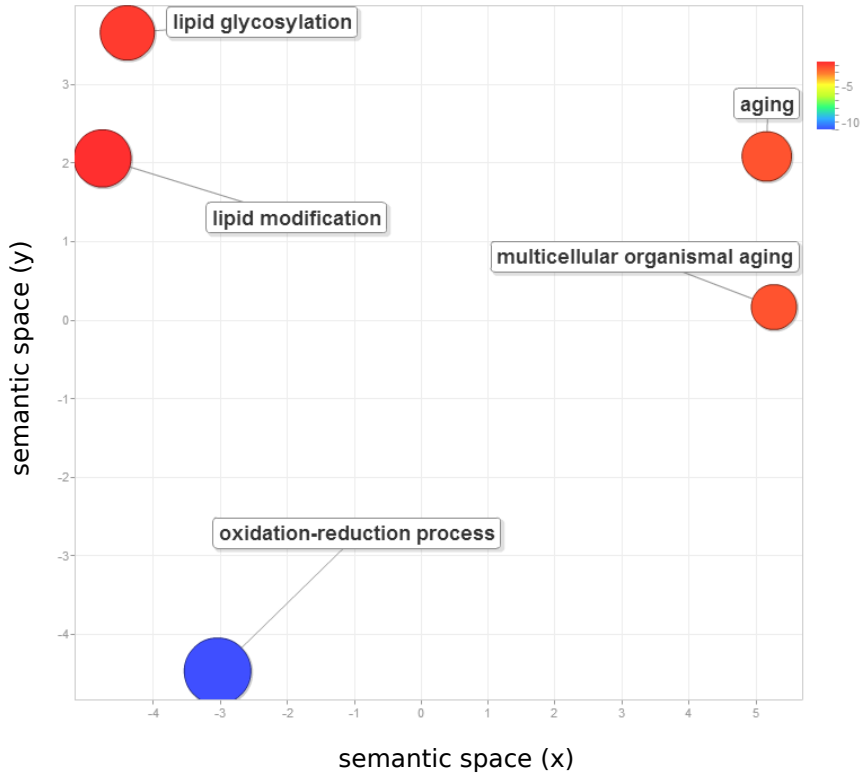


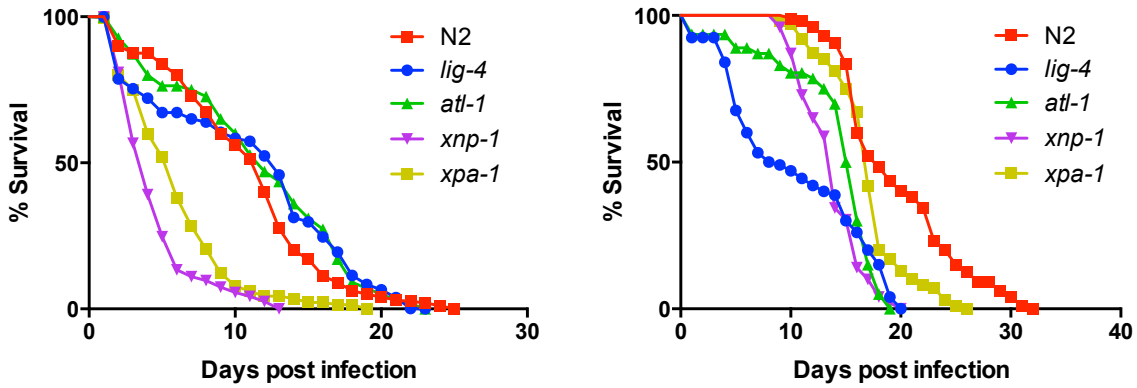
Figure 4.16: Functional classification of genes common to the cellular stress and *E. faecium* infection gene signatures. The GO terms enriched in the set of 49 genes represented in the overlap in Figure 4.15, part F, using the REViGO visualization tool. Colors corresponding to $\log_{10}$ p-value of the GO term enrichment are provided in the legend.

Moreover, these 49 genes showed an enrichment in cytochrome P450s, alcohol dehydrogenases, glutathione S-transferases, and UDP-glucuronosyl/ UDP-glucosyltransferases, all of which are phase I and phase II detoxification genes (Table 4.5).

Table 4.5: Enrichment of protein domains in genes induced by *Enterococcus* and organismal stress, based on InterPro domains.

InterPro Term	Description	Count	% enrichment	*p*-value
IPR017972	Cytochrome P450, conserved site	8	10.26	4.43×10^{-8}
IPR002328	Alcohol dehydrogenase, zinc-containing, conserved site	3	3.85	4.21×10^{-4}
IPR004046	Glutathione S-transferase, C-terminal	4	5.13	1.51×10^{-3}
IPR002213	UDP-glucuronosyl/UDP-glucosyltransferase	4	5.13	6.15×10^{-3}

Previously, when looking at the gene families enriched in the *E. faecium* infection gene signature, I noticed a strong enrichment in genes related to DNA metabolism and DNA repair (Table 4.6). This was especially intriguing, as all the cells in the *C. elegans* soma are post-mitotic, and as a result, DNA repair primarily occurs in the *C. elegans* gonad. These DNA repair genes, however, did not fall into one particular DNA repair pathway, but represented many different pathways, including non-homologous end joining, mismatch repair, nucleotide excision repair, and homologous recombination. I wondered whether DNA repair pathways played a protective role in the *E. faecium* infection and tested this possibility by assaying mutants deficient in four key DNA repair genes: *lig-4* (non-homologous end-joining, *atl-1* (critical for chromosomal segregation), *xnp-1* (ATR-X homolog), and *xpa-1* (nucleotide excision repair) for their survival on *Enterococcus*. When these mutants were tested for their sensitivity to *E. faecalis* infection, *xpa-1* and *xnp-1* exhibited a severely immunodeficient phenotype (Figure 4.17). In contrast, in the case of *E. faecium* infection, *lig-4* and *atl-1* were particularly sensitive, as the lifespan differences between these mutants and wild-type N2 reached statistical significance; however, this was not the case for these same mutants when infected with *E. faecalis*. The reason for the differential susceptibilities of the mutants to each enterococcal species



(a) *E. faecalis* **(b)** *E. faecium*

Figure 4.17: Role of DNA repair genes in *C. elegans* defense against *Enterococcus*. Effect of *Enterococcus* infection on wild-type N2 and mutant *lig-4*, *atl-1*, *xnp-1*, and *xpa-1* worms. Worms deficient in *xnp-1* and *xpa-1* showed a statistically significant decrease in lifespan, as compared to wild-type N2 worms, in both *E. faecalis* and *E. faecium* infection ($p < 1.0 \times 10^{-10}$ for all comparisons). The *atl-1* and *lig-4* mutants showed a statistically significant decrease in lifespan when infected with *E. faecium* ($p < 1.0 \times 10^{-10}$ and $p = 3.0 \times 10^{-4}$, respectively) but not *E. faecalis* ($p = 0.06$ and $p = 0.05$, respectively).

remains unclear, but it is possible that DNA repair is a nexus at which immune pathways and stress signals can meet.

Given that a variety of DNA repair proteins are upregulated in an *E. faecium* infection, I speculated that *E. faecium* itself may induces DNA damage directly, perhaps through the production of reactive oxygen species such as hydrogen peroxide or superoxide. This was an appealing possibility, as there is evidence for the production of ROS from intestinal epithelial cells. This might explain other signatures I observed (namely, oxidation-reduction, lipid metabolism, and cofactor binding) and the significant overlap between stress gene signatures and the *E. faecium* gene signature.

I tested the role of hydrogen peroxide in the pathogenicity of the *E. faecium* infection, as it had been established that under anaerobic conditions, *E. faecium* could produce hydrogen peroxide, which could kill *C. elegans*[139]. Unpublished data (Danielle Garsin, personal communication) also suggested that *E. faecium* could even produce hydrogen peroxide in aerobic conditions, although at a much lower level. To examine whether hydrogen peroxide production contributed significantly to

Table 4.6: *C. elegans* DNA repair genes differentially upregulated during *E. faecium* infection
Abbreviations: NER, nucleotide excision repair; HR, homologous recombination; NHEJ, non-homologous end-joining.

Public name	Gene name description	Pathway/Function
apn-1	APurinic/apyrimidinic endoNuclease	NER
atl-1	ATM(ataxia telangectasia mutated)-Like	HR
C44E4.1	C44E4.1	
cku-80	Caenorhabditis KU	NHEJ
C14A4.4	Cell-death-Related Nuclease	
Y37B11A.2	DNA polymerase	
T24C4.5	DNA primase	
A55A3.3	FACT complex subunit spt-16	
gei-17	GEX Interacting protein; SUMO ligase; telomere maintenance	
hmg-3	HMG	
lig-4	LIGase	NHEJ
msh-4	MutS Homology family	HR
polk-1	POLK (DNA polymerase kappa) homolog	Methylation damage
polq-1	POLQ (DNA polymerase theta) homolog	Interstrand cross-links
R05D3.12	Putative DNA topoisomerase 2, mitochondrial	
M03C11.8	Putative SMARCAL1-like protein; Protein archease-like	
rev-1	REV1 (translesion DNA polymerase) homolog	
rcq-5	ReCQ DNA helicase family	
top-3	TOPoisomerase	HR
trt-1	Telomerase Reverse Transcriptase	
xpg-1	XPG (Xeroderma Pigmentosum group G) DNA repair gene homolog	NER
spo-11	homolog of yeast SPOrulation gene; nuclear export receptor	
xnp-1	human XNP gene related; helicase	
dna-2	yeast DNA helicase/endonuclease family	HR

pathogenicity, I assayed the lifespan of worms fed *E. faecium* E007 strain, with or without catalase, and found no difference between the two groups (Figure 4.18). However, addition of catalase did appear to be important for the virulence of a different *E. faecium* strain called 1,231,502. While it is possible that E007 *E. faecium* does not produce hydrogen peroxide to cause ROS and DNA damage, it is also conceivable that *E. faecium* only produces low levels of hydrogen peroxide that only wild-type worms, with intact immune and stress responses, can tolerate. If this were true, when one

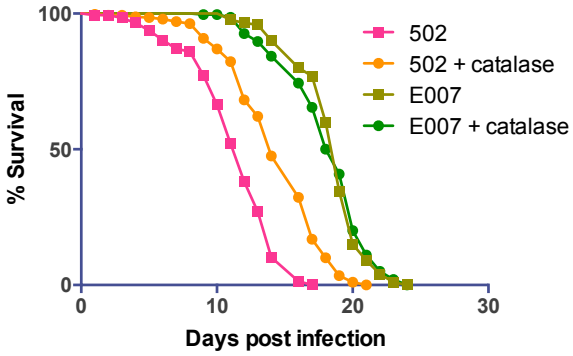


Figure 4.18: Catalase offers no protective effect to worms infected with E007 *E. faecium* infection. Survival of *fer-15;fem-1* worms on two strains of *E. faecium*, strain E007 (used for the majority of this study) and strain 1,231,502, and the effect of catalase on *E. faecium* pathogenicity. Catalase extends worm lifespan on the "502" strain ($p < 1 \times 10^{-10}$), but not on E007 ($p = 0.12$), suggesting that hydrogen peroxide production is irrelevant in pathogenicity in E007.

of these immune or stress response pathways is removed, the protective effect of adding catalase to *C. elegans* infected with E007 *E. faecium* could be unmasked. Alternatively, it is also possible that even though DNA repair genes are differentially upregulated in the *E. faecium*-infected *C. elegans*, DNA repair itself is not taking place. Instead, DNA damage genes expressed in the gonad may be part of the defense response indirectly, perhaps by relaying a signal to the soma to activate immune and stress response pathways.

4.2 Discussion

While *E. faecalis* is the most common enterococcal species responsible for nosocomial infections, *E. faecium* infections have been increasing steadily in the past decade. In spite of this, *E. faecium* virulence mechanisms and their effects on the host remain poorly understood. Previously, it had been shown that in the *C. elegans* infection model, *E. faecium* proliferates in and distends the host intestine, but does not kill the nematodes. This current study provides evidence that *E. faecium* is pathogenic in *C. elegans* and activates host stress and immune pathways. Colonization of the *C. elegans* intestine with *E. faecium* triggers a rapid transcriptional defense response dependent upon known immune signaling pathways and stress response pathways, and suggests an overlap in the host stress and im-

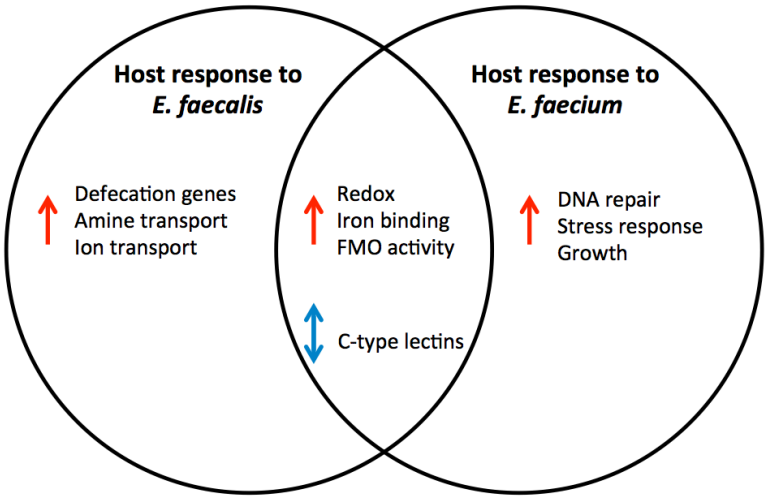


Figure 4.19: The *C. elegans* host response to *E. faecalis* and *E. faecium*. A summary of some of the transcriptional profiling experiments of this study.

mune response pathways to ultimately contribute to pathogen resistance.

4.2.1 Perception of damage, DNA repair and detoxification programs

Using ultrastructural imaging, I confirmed that *E. faecium*, like *E. faecalis*, proliferates in and distends the *C. elegans* intestine in the absence of any obvious host damage. Despite the ability of *E. faecium* to induce such a dramatic physiological change (intestinal distention) in *C. elegans*, it is interesting to note that, in contrast to *E. faecalis*, intestinal distention alone does not substantially impair the defecation rhythm of *C. elegans* (Figure 3.3). It is possible that alterations in a particular part of the *C. elegans* alimentary system (perhaps at the most distal end) are what determines whether the worm will be unable to defecate normally or not. As our transmission electron microscopy images are of the mid-body, I was not able to see the section of the intestine near the anus, and it will be worth imaging *E. faecalis* and *E. faecium*-infected worms with fluorescently-labeled bacteria and differential interference contrast microscopy of the *C. elegans* distal intestine to examine whether *E. faecalis*-infected worms are more distended in the posterior of the worm than *E. faecium*-infected worms. An alternative explanation might be that *E. faecium* lacks a virulence factor that is encoded

in *E. faecalis* to interfere with the host defecation motor program. Additionally, it is possible that *E. faecium* causes minor damage in *C. elegans* in the absence of an intact immune response, as *E. faecium*-infected *pmk-1* worms have an altered defecation rhythm. Studies using microscopy to assess any potential differential distension and damage in the posterior intestinal cells between wild-type N2 and *pmk-1* mutant worms will be necessary to evaluate this possibility.

To assess whether an active immune response was required for *C. elegans* defense against *E. faecium*, I tested a number of immune signaling mutants for their susceptibility to *E. faecium* and found that mutants deficient in PMK-1, BAR-1, or FSHR-1 signaling were susceptible to infection with *E. faecium*, despite wild-type worms exhibiting no shortening of lifespan on *E. faecium*. This result is reminiscent of what had been observed in the treatment of *C. elegans* with exotoxin ToxA[125], a protein produced by *P. aeruginosa* that inhibits protein translation by ADP-ribosylation of elongation factor-2. Wild-type *C. elegans* fed ToxA-expressing *E. coli* exhibited no alteration in lifespan, but worms deficient in the *pmk-1*, *fshr-1*, or *zip-2* pathways were hypersensitive to ToxA, suggesting that immune pathways are required for defense against ribotoxin. Thus, it is possible that *E. faecium*, like *P. aeruginosa*, produces a molecule that induces damage in the host cells, which wild-type worms can manage and detoxify, but immune mutants cannot.

In our transcriptional profiling of *E. faecium*-infected worms, I identified a strong signature of DNA repair response, xenobiotic metabolism and fatty acid metabolism. The activation of genes normally responsive to DNA damage was especially exciting, as *E. faecalis* has been shown to produce superoxide and hydrogen peroxide directly through autooxidation of membrane-associated demethylmenaquinone[72]. (There have been no reports on the ability of *E. faecium* to produce superoxide.) In one study, epithelial cell lines co-incubated with *E. faecalis* showed more DNA damage with a wild-type *E. faecalis* strain of OG1RF than with a transposon-inactivated mutant with attenuated extracellular superoxide production. On the other hand, *E. faecalis*-mediated DNA damage could be prevented by catalase, but not manganese superoxide dismutase, implicating hydrogen peroxide as the genotoxin. The effect of superoxide production was also recapitulated in an *in vivo* rat

model of *E. faecalis* intestinal colonization[72,232]. Furthermore, an independent study demonstrated that *E. faecalis* infection induces intracellular ROS production through a pathway independent of oxidative phosphorylation to induce mitochondrial DNA instability[203].

If *E. faecium* is similar to *E. faecalis* in its ability to produce ROS, perhaps the ROS produced by *E. faecium* in the *C. elegans* killing assay is at a level that can be tolerated by wild-type worms, with intact immune and stress response pathways. I showed that the addition of catalase to *E. faecium*-infected worms had no effect on *E. faecium* pathogenicity in the case of E007, suggesting that either *E. faecium* strain E007 does not produce hydrogen peroxide, or that the levels of hydrogen peroxide are low enough that *C. elegans* catalases can break it down efficiently. I did not test whether catalase partially rescues *E. faecium*-infected worms deficient in PMK-1, BAR-1, or FSHR-1, though such a finding would suggest that these immune pathways may be required for induction of genes responsive to the decomposition of hydrogen peroxide into water and oxygen. Additionally, I did not test the addition of manganese superoxide dismutase to *E. faecium*-infected *C. elegans*, and it may be possible that this strain of *E. faecium* produces superoxide as a primary genotoxin, and not hydrogen peroxide.

Our data does not exclude the possibility that any potential ROS produced during *E. faecium* infection of *C. elegans* is actually derived from the host. It has been demonstrated that ROS are generated during bacterial infection (including infection with *E. faecalis*) in *C. elegans* by the dual oxidase BLI-3 (homolog of Duox1), which activates a protective SKN-1 response via the PMK-1 p38 MAPK cascade[221]. The production of ROS by *C. elegans* was found to have a protective role, suggesting that *C. elegans* intestinal cells generate extracellular ROS as an antimicrobial response, akin to the respiratory burst in mammalian neutrophils. To protect from collateral damage, these same intestinal cells produce intracellular antioxidants (like superoxide dismutases and catalases) for protection against the damaging effects of the ROS produced. In our system with *E. faecium* infection, it is possible that *E. faecium* activates BLI-3 to produce superoxide. To better understand the role of ROS in our enterococcal infection system, it will be worthwhile staining *E. faecalis*- and *E. faecium*-infected *C. elegans* with a ROS-sensitive dye, which may offer clues on whether the host, pathogen, or both are

producing ROS.

E. faecium may also be able to indirectly cause the production of ROS within the *C. elegans* host. From transcriptional profiling data, *E. faecium* induces a strong detoxification response because it produces toxins or xenobiotics, a hypothesis that will need to be tested by metabolically profiling *E. faecium* alone, along with *E. faecium*-infected worms. It is possible that during the detoxification of *E. faecium*-derived xenobiotics in *C. elegans*, ROS is produced, as the catabolism of certain xenobiotics by cytochromes has been demonstrated to enhance ROS production. The increased ROS levels, in addition to damaging host proteins, lipids and membranes, also induces DNA damage. Phase 2 detoxification reactions may be acting to protect *C. elegans*, especially the germline, which, unlike the soma, is especially susceptible to DNA damage. The germline then responds with the upregulation of DNA repair genes to counter and repair the DNA damage.

It will also be critical to assess whether *Enterococcus* infection of *C. elegans* can cause DNA damage. One indirect way of addressing this is by examining the progeny of *E. faecalis*- and *E. faecium*-infected *C. elegans* for an enrichment in males versus a heat-killed *E. coli*-fed control, as *C. elegans* males are generated by chromosomal non-disjunction[7]. A more direct test for DNA repair is by immunostaining *C. elegans* gonads for RAD-51, a *C. elegans* protein that binds the ends of DNA at double-stranded breaks, an assay that is similar to the staining of H2AX in mammalian cells[23]. This experiment would help distinguish between two models, one in which *C. elegans* is responding to DNA damage, and another in which *C. elegans* responds to some facet of pathogen infection in the same way that it responds to DNA damage. If no DNA damage can be observed, what would the upregulation of these DNA damage proteins mean? Does *C. elegans* responds to *E. faecium* in the same way that it responds to DNA damage, or could it be that there is an overlap between immune or stress signaling pathways and pathways that respond to DNA damage? Future work will be required to differentiate between these various possibilities.

4.2.2 Stress response pathways in *E. faecium* infection

The role of stress response pathways in pathogen resistance has been underappreciated until recently. In one example, a role for SKN-1 has been demonstrated in mitigating the collateral damage caused by the host's production of extracellular ROS, made by the *C. elegans* intestinal cell to target bacteria in the gut[221]. The host response to ER stress, called the unfolded protein response, has also been shown to have a protective role during infection of *C. elegans*. It is now appreciated that the activation of PMK-1 in response to *P. aeruginosa* infection induces the XBP-1-dependent unfolded protein response, relieving the ER stress caused by the host's massive secretory response to microbes and abiotic toxins[178]. Additionally, it is now recognized that the *C. elegans* mitochondrial unfolded protein response is activated by and protects against *P. aeruginosa* infection, perhaps suggesting a means by which host cells can detect pathogens that damage mitochondrial function[157]. Furthermore, an independent study using *P. aeruginosa* infection of *C. elegans* demonstrated that pyoverdin, a small iron-chelating molecule secreted by *P. aeruginosa*, disrupts mitochondrial homeostasis and triggers mitophagy, a process that confers protection to *C. elegans* against *P. aeruginosa*[98].

I discovered in my study that *E. faecalis* and *E. faecium* are able to induce a very similar set of *Enterococcus*-activated genes, and that the previously identified immune pathways are required for the induction of these genes in the case of both *E. faecalis* and *E. faecium* infection. However, in response to infection with *E. faecium*, the induction of a significant subset of these genes requires at least two stress-induced genes, KGB-1 and SKN-1. Using RNAi to knockdown these several stress response pathway genes, I found that the absence of the JNK-like MAPK KGB-1 (which regulates the response to stress by heavy metal, protein folding, and pore-forming toxins), or MDT-15 (which regulates the response to oxidative stress and xenobiotics) severely compromised *C. elegans* to infection with *E. faecium* and *E. faecalis*. As discussed below, through comprehensive analysis in characterizing the transcriptional profiles of these various *C. elegans* mutants, I revealed a previously uncharacterized and underappreciated role for the function of stress response pathways in the *C. elegans* defense

response.

As alluded to previously, KGB-1, in addition to protecting *C. elegans* from heavy metal and protein folding stress, also mediates resistance to the pore-forming toxin Cry5B, a member of the three-domain Cry toxin family of *Bacillus thuringiensis*; moreover, KGB-1 was demonstrated to be a master regulator of the Cry5B pore-forming toxin-induced transcriptional response, controlling half of the genes induced by Cry5B. These lines of evidence support the idea that, in addition to the other cellular processes, *C. elegans* also monitors membrane integrity within its cells; indeed, perturbation of membrane integrity is one of the theorized patterns of pathogenesis monitored by the host. The KGB-1 pathway is also required for aversion behavior in response to pathogenic microbes and gene inactivations of essential cellular pathways, implicating its role in cellular surveillance. From the work described in this thesis, I suspect that KGB-1 may also be important in the integration of several different stress-related signals; one possibility is that *E. faecium*, and even *E. faecalis*, may produce an uncharacterized toxin or virulence factor during infection that compromises membrane integrity.

As previously discussed, SKN-1 was first identified for its role in the *C. elegans* response to oxidative damage and xenobiotic stress, though now it has also been demonstrated to have a central role in the response to ER stress, where it regulates an ER stress gene network distinct from its oxidative stress response, requiring ER-associated factors to "license" SKN-1 to defend against oxidative stresses[53]. Additionally, SKN-1 has also been shown to coordinate the *C. elegans* response to fasting by coupling proline catabolism and lipid utilization[153]; thus, it appears that SKN-1 may orchestrate several different stress responses to balance between cellular functions and stress responses. While worms treated with RNAi against *skn-1* are unable to upregulate stress-responsive genes after infection with *E. faecium*, I see little to no difference in the survival of worms treated with *skn-1* RNAi versus the vector control. Even though a hypersusceptibility phenotype had been reported for worms deficient in *skn-1*, this result may be the result of different RNAi clones being used to knock down *skn-1* in these studies. In future work, it will be necessary to re-test susceptibility on *Enterococcus* using a null mutant of *skn-1*.

While SKN-1 is critical for the induction of many stress-responsive genes, it does not accomplish this on its own; rather, it requires the transcriptional co-regulator MDT-15, a subunit of the conserved Mediator complex to activate genes in response to oxidative stress[54]. The roles of MDT-15 are in the regulation of the oxidative stress response (partnering with SKN-1), and fatty acid metabolism (where it interacts with the SREBP homolog SBP-1 and nuclear hormone receptor NHR-49); these two roles are functionally separate[209]. More recently, however, MDT-15 has been shown to have a protective role against infection with *P. aeruginosa* by upregulating a number of detoxification genes downstream of PMK-1; additionally, MDT-15 mediates a protective role against phenazines, a family of small, secreted, toxic molecules produced by *P. aeruginosa*[169]. The role of MDT-15 in *Enterococcus* infection had not yet been examined in the context of *Enterococcus* infection of *C. elegans* until this study. Given that knocking down *mdt-15* causes susceptibility to *E. faecalis* and *E. faecium* infection, one would surmise that MDT-15 may play a protective role in *Enterococcus* infection by transcriptionally regulating *C. elegans* defense genes, especially detoxification-related genes, many of which are enriched in the *Enterococcus* gene signature. Given that MDT-15 acts downstream of PMK-1 to modulate a number of defense response genes in response to *P. aeruginosa*, it will be important to use the NanoString multiplexed gene expression system to profile the *mdt-15* in *Enterococcus* infection to see whether its gene expression pattern clusters more closely to that of other *Enterococcus*-infected stress mutants or to that of *Enterococcus*-infected immune mutants, especially *pmk-1*. Furthermore, it will be important to perform epistasis anaylsis using the *pmk-1* and *mdt-15* mutants in infection with *E. faecalis* and *E. faecium*.

While I expected to see the role of the KGB-1 and SKN-1 stress response pathways in gene induction correlate with their role in resistance to *Enterococcus*, it is important to note that gene induction was assessed at a timepoint early in infection (8 hours), which may not reflect the same set of genes that *C. elegans* upregulates late in infection. Because *Enterococcus* infections span several days, it is possible that these stress-related pathways are important in the regulation of many genes that have a protective role in defense, at later stages of the infection. For that reason, *skn-1* may regulate a

set of stress-responsive genes early in infection, which are critical at that time point, but perhaps they ultimately are turned on later in infection by other factors (functional redundancy with other stress-related transcription factors), and for that reason, the *skn-1* mutant is not extremely sensitive to *Enterococcus* infection.

4.2.3 The role of nuclear hormone receptors in immunity

As discussed previously in this chapter and in the Introduction to this thesis (Chapter 1), nuclear hormone receptors are a family of transcription factors, regulated by small lipophilic hormones, that integrate numerous environmental cues to modulate the transcriptional response to pathogenic infection. While the human, mouse and *Drosophila* genomes contain 48, 49 and 18 predicted NHRs, respectively, *C. elegans* contains a greatly expanded family of NHRs with 284 predicted members [118, 199]. However, only 15-20 *C. elegans* NHRs are conserved among metazoans [119,222], suggesting that the expansion of *C. elegans* NHRs may not have simply resulted in functional redundancy, but that the evolution of NHRs demonstrates species-specificity to recognition of xenobiotics. Little is known about the ligands of the nuclear hormone receptors in *C. elegans*, with NHR-8 being the sole receptor with a known function in xenobiotic metabolism, mediating resistance resistance to chloroquine and colchicine [107].

From *C. elegans* studies on the role of nuclear hormone receptors in pathogen resistance, two receptors have been identified that negatively regulate pathogen resistance. One nuclear hormone receptor, DAF-12, was demonstrated to negatively regulate *P. aeruginosa* resistance through the regulation of the *let-7* family of microRNAs via *mir-84* and *mir-241*, which directly target SKN-1 downstream of PMK-1 [109]. In another study, exploring the role of nuclear hormone receptor NHR-25, it was demonstrated that inactivation of *nhr-25* increased the susceptibility of *C. elegans* to infection with the fungal pathogen *D. coniospora* and increased *D. coniospora* spore adherence. Somewhat paradoxically, the *nhr-25* mutant constitutively expressed antimicrobial peptides, including *nlp-29*, a peptide induced upon wounding of the cuticle and infection by *D. coniospora*. The most likely ex-

planation for these phenotypes of the *nlp-29* mutant is that because NHR-25 is also an important transcriptional regulator for molting genes, the *nhr-25* mutant exhibits a weak cuticle barrier phenotype. As a result, the worm constantly perceives itself as being wounded, and thus upregulates antimicrobial peptides constitutively. However, the upregulation of these antimicrobial peptides is not sufficient for defense against *D. coniospora*; in fact, it is likely that *D. coniospora* uses the *nhr-25* mutant's leaky cuticle to kill the worm faster.

In my study, I identified three NHRs that were upregulated in infection with either one or both enterococcal strains tested: NHR-68, NHR-101, and NHR-114. Unlike the previously described NHRs, the NHRs I have identified are positive regulators of pathogen resistance. No known phenotypes are associated with mutants or RNAi-treated worms corresponding to these NHR genes, save for NHR-68, which when knocked down, causes an increase in fat content. I was able to show that worms deficient in any of these NHRs were hypersusceptible to *E. faecalis* infection, though only the worms deficient in *nhr-68* and *nhr-101* had a higher susceptibility to *E. faecium* infection. This was an interesting result, as most of the previous mutants tested previously, had increased susceptibility to infection with both enterococcal species. Thus, NHRs may help to explain the differential response of the host to *E. faecalis* and *E. faecium* infection. These data may support the idea that nuclear hormone receptors are sensors that "tune" the necessary stress responses, depending on the type of pathogen or xenobiotic present, though this possibility remains to be investigated.

It has been speculated that the mediator subunit MDT-15, in addition to interacting with NHR-49 and the sterol response element binding protein SBP-1 in fatty acid metabolism[209,236], may interact with NHRs during xenobiotic regulation, as other mammalian Mediator subunits have been shown to mediate detoxification through the NHRs PXR and CAR[86,123]. This may explain why the *mdt-15* mutant is extremely sensitive to pathogen infection, as it serves as a node for many different nuclear hormone receptors in *C. elegans*. In future studies, it may be worth performing an RNAi screen to search for other nuclear hormone receptors that positively regulate pathogen resistance, as such experiments may eventually lead to the characterization of host- or pathogen-derived ligands, which

may include xenobiotics, that are recognized by *C. elegans*.

4.2.4 The role of *C. elegans* small RNA processing in stress and immunity

In my study, I demonstrated that worms deficient in small RNA processing were impaired in their ability to induce stress-responsive genes after infection with *E. faecium*. Additionally, worms for which *drsh-1* and *dcr-1* were knocked down were hypersusceptible to infection with either *E. faecalis* or *E. faecium*. These findings link the *C. elegans* small RNA processing pathway with stress and pathogen resistance. There is already evidence for this connection. One study demonstrated that *dcr-1* mutant alleles that were deficient in miRNA processing, but not those deficient only in RNAi, were resistant to a virulent strain of *Bacillus thuringiensis* – a finding that perhaps implies that the increased resistance of *dcr-1* mutants to *B. thuringiensis* is due to the upregulation of pathogen response or stress genes[75]. In a different study (with nearly the opposite conclusion about DCR-1 and its role in stress and longevity), *dcr-1* mutants had a reduced lifespan and stress tolerance, due to their inability to process the miRNA *lin-4*[136]. In future studies, it would be of interest to see whether *lin-4* or the two other miRNAs that are controlled by intestinally-expressed Dicer (*let-7* and *miR-231*) also play protective roles in *Enterococcus* infection; furthermore, identifying the targets of the relevant miRNAs in pathogen resistance would also be of interest, and these may be detoxification genes or antibacterial factors.

As discussed previously, small RNAs have long been implicated in *C. elegans* defense against viruses and other sources of genomic modification. In plants, host endogenous small RNAs comprise one of the most robust strategies plants have evolved to mount an immune response against bacterial infection and mediate disease resistance. These results on the role of small RNA processing in pathogen resistance add to the already growing body of literature of small RNA control of immune-related genes.

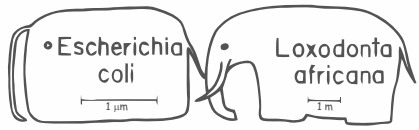
4.2.5 Crosstalk between the stress and immune responses

This study is the first to link the leucine-rich repeat G-protein coupled receptor, FSHR-1, to a stress response. FSHR-1 is required for resistance to *P. aeruginosa*[163], *E. faecalis*, and now *E. faecium*, and has been shown in previous studies to act in parallel to DAF-2 and the p38 MAPK PMK-1, mediating its role in pathogen resistance in the *C. elegans* intestine. The ligand for FSHR-1 is unknown; by homology, its canonical ligand is predicted to be the heterodimeric glycopeptide hormone FSHα/β, but worms lack the FSHα subunit and the gene that is most similar to FSHβ does not appear to have a role in pathogen resistance. It is tempting to speculate that FSHR-1 may be activated by a noncanonical ligand, perhaps derived from the host (akin to Spätzle in *Drosophila*), in response to an upstream signal from a pattern recognition receptor. In our studies, I see that FSHR-1 regulates the induction of the same *Enterococcus*-activated genes that KGB-1 and SKN-1 regulate. It may also be possible that FSHR-1 senses a noncanonical ligand in response to an upstream signal of damage, akin to the activation of DCAR-1 in the hypodermis[246]. Future studies probing the role of FSHR-1 will be necessary to determine this; it will be useful to perform a microarray comparing WT and *fshr-1* worms, at steady-state and during infection, to identify and study the genes that comprise the FSHR-1 targets. Concomitantly, it will be worth examining whether FSHR-1, or any of the other immune mutants that have been characterized, are compromised in the response to cellular stress (including ER stress, mitochondrial stress, oxidative stress, membrane integrity stress by pore-forming toxins, heavy metal stress, and DNA damage).

In the experiments described in this chapter, I provide further evidence that the host differentiates between *E. faecalis* and *E. faecium* by sensing the impact of the infection on host cellular physiology, highlighting the crosstalk between sensing of infectious microbes and host damage in the immune system. The results in Chapter 3 demonstrate that heat-killed *Enterococcus* activates a defense response in *C. elegans*, and that pre-treatment with heat-killed *Enterococcus* protects *C. elegans* from a subsequent infection with *E. faecalis*, supporting a model that the perception of MAMPs can

protect *C. elegans* from microbial infection. Given the results in Chapter 4 that suggest crosstalk between MAMP recognition, stress responses, and host cellular damage, it is possible that the sensation of DAMPs can cause *C. elegans* to enter a state of heightened surveillance, rendering *C. elegans* more resistant to microbes. Further studies will required to dissect and unravel the network of host-protective mechanisms, including immunity, xenobiotic detoxification, and oxidative stress response, that protect the worms from pathogenic infection.

What is true of E. coli is also true of the elephant.



Jacques Monod

5

Conclusions and future directions

When faced with a pathogen infection, a host must coordinate a multifaceted response to tolerate the myriad physiological challenges resulting from the infection. The host immune system, in addition to producing immune effectors that directly kill the pathogen, must also initiate stress responses, which protect the host not only from the effects of toxins derived from the pathogen, but also from the collateral damage that host cells may suffer, either directly or indirectly, from its defense response against the pathogen. Thus, it is extremely critical that host immune effector mechanisms are tightly linked to cellular repair processes, as uncontrolled activation or inappropriately timed repair processes can lead to further host damage or even death.

In this thesis, I have explored these concepts, using *Enterococcus* infection models of *C. elegans* to probe the host response to bacterial infection. Although prior work had shown that *Enterococcus* was capable of infecting *C. elegans* and several virulence factors in *E. faecalis* had been implicated in *E. faecalis* pathogenesis, little was known about enterococcal infection biology in *C. elegans*. Furthermore, it was not understood why *E. faecalis* and *E. faecium*, which both distend the *C. elegans* intestine, differentially affect the lifespan of *C. elegans* during infection.

The work presented in Chapter 3 in part confirms previous findings that *E. faecalis* and *E. faecium* both cause intestinal distention in *C. elegans* but extends this work by using ultrastructural imaging, which shows that this distention, even late in infection, appears to occur in the absence of any severe host damage, in contrast to what has previously been observed in infections of *C. elegans* with *P. aeruginosa* or *S. aureus*. Additionally, I demonstrate that unlike infection with *E. faecium*, *E. faecalis* infection lowers pharyngeal pumping rates and defecation rates in *C. elegans*, and that this effect is abrogated when *E. faecalis* is heat-killed, a result that suggests that bacterial infection alters the physiology and behavior of *C. elegans*.

Through whole-genome transcriptional profiling and analysis of *E. faecalis*-infected *C. elegans*, I identified 270 *E. faecalis*-upregulated genes that comprise the *E. faecalis* infection signature, which is enriched in UDP-glucosyltransferases and cytochrome P450 genes, a finding that suggests that *E. faecalis* infection induces a potent detoxification response in *C. elegans*. Furthermore, genes associated with growth, adenyl nucleotide binding, oxidation-reduction, electron carrier activity, and carbohydrate binding, are also over-represented among the *E. faecalis*-activated genes.

I present data to show that *E. faecalis* activates known *C. elegans* immune signaling pathways, including the PMK-1 p38 MAPK pathway and the BAR-1 β-catenin pathway, and that these pathways are required for a wild-type defense against *E. faecalis*. This study also identified new gene targets of previously studied immune pathways (such the PMK-1, FSHR-1, and BAR-1 pathways), an important finding, as the genes regulated by these pathways in response to Gram-positive pathogens are different from those identified in the response to the Gram-negative pathogen, *P. aeruginosa*. By

comparing the transcriptional profiles of *C. elegans* fed *E. faecalis* to the profiles of worms fed with *B. subtilis*, *E. faecium*, *S. aureus*, or *M. nematophilum*, I attempted to identify genes that comprise a Gram-positive bacterial infection signature; however, it appears that the infection gene signatures for Gram-positive bacterial infections are actually quite disparate.

Because both *E. faecalis* and *E. faecium* elicit the induction of a similar set of genes in *C. elegans*, I hypothesized that MAMPs common to enterococcal species may be triggering the *C. elegans* host response. I then demonstrated that exposure of *C. elegans* to either heat-killed *E. faecalis* or *E. faecium* elicited similar transcriptional responses to that of live infection by either enterococcal species, and that this induction was abrogated in the absence of PMK-1 or BAR-1. Furthermore, I discovered that pre-exposure to heat-killed *Enterococcus* renders *C. elegans* a protective advantage against later infection with live *Enterococcus*.

In Chapter 4, I characterize the *E. faecium* infection of *C. elegans*, for which very little was known, and provide evidence that *C. elegans* deficient in PMK-1, BAR-1, or FSHR-1, exhibit a dramatically shortened lifespan when infected with *E. faecium*, a finding that unexpectedly showed that *E. faecium* is a *C. elegans* pathogen, and that an active host defense response is required to keep an *E. faecium* infection at bay. Using whole-genome transcriptional profiling, I probed the *C. elegans* host response to *E. faecium* and identified an enrichment in genes associated with DNA metabolism and the response to DNA damage stimuli. The *E. faecium* infection gene signature was shown to contain a number of immune effectors (*e.g.*, C-type lectins), as well as phase I and II detoxification enzymes.

I then analyzed the gene expression profiles of a set of *C. elegans* mutants deficient in immune signaling using a multiplexed gene expression platform for 67 *Enterococcus*-regulated genes and 5 housekeeping genes and determined that the transcriptional pattern for all the mutants was very similar to that identified for *E. faecalis*-infected *C. elegans*. Intriguingly, however, while both enterococcal species induce the same defense response genes to similar levels, the regulation of these genes is controlled by different stress response pathways. Specifically, during *E. faecium* infection, I identified a specific subset of host effectors dependent upon the *C. elegans* stress-associated pathways SKN-1

and KGB-1. In contrast, the induction of this same set of host effectors by *E. faecalis* is regulated independently of SKN-1 and KGB-1. Additionally, the *C. elegans* small RNA processing pathway, which had previously been implicated in the response to oxidative stress, was also required for the full induction of this specific subset of host effectors. Finally, the LRR-containing GPCR FSHR-1 also plays a key role in regulating the the induction of this subset of stress-related genes, although this regulation takes place in both *E. faecalis* as well as *E. faecium* infection, potentially implicating FSHR-1 in the response to cellular stress.

In summary, these results show that *C. elegans* is a useful model for studying the host-pathogen interactions from the perspectives of both the host and pathogen. My findings have important implications for Toll-independent innate immune mechanisms and provide evidence for the ability of *C. elegans* to potentially respond to MAMPs in a manner dependent upon previously identified immune signaling pathways. These results revise our previous notions on pathogen recognition in *C. elegans* and provide preliminary evidence for potential "immune memory" in invertebrates, in the sense that it can protect from adverse consequences of infection. Furthermore, we also revise the notion that *E. faecium* is not a pathogen in *C. elegans* and provide evidence for the underlying differences in host response and, potentially, virulence mechanisms between *E. faecalis* and *E. faecium* infection in the *C. elegans* host model. The paradigm of extensive crosstalk between conserved pathways that sense MAMPs, xenobiotics, and host stress observed in this thesis is likely to apply to metazoans in general.

5.1 Future directions

The results of the experiments described in this thesis provide evidence that MAMPs may be recognized by the *C. elegans* immune system – shown by the activation of the F35E12.5 transcriptional reporter with heat-killed *B. subtilis* (Appendix B), as well as the activation of a protective response by heat-killed *E. faecalis* (Chapter 3) – raising intriguing questions that are worth future investigation. One potential path of investigation would be pursuing the identification of the bacterial products that

are involved in the induction of this protective *C. elegans* response using a biochemical approach. In *C. elegans* there are other examples of single molecules and proteins that are sufficient for the induction of various transcriptional immune reporters, such as the expression of *P. aeruginosa* ribotoxin ToxA for the induction of the infection responsive gene-1 (*irg-1*) [125] and the small molecule RPW-24 for the induction of the *P. aeruginosa*-induced gene *F08G5.6* [169].

Another main idea explored in this thesis is the relationship between cellular stress responses and immunity. *clec-60* appears to be an *Enterococcus*-activated gene induced by both live and heat-killed *E. faecalis*. Furthermore, *clec-60* appears to be regulated by stress response pathways (FSHR-1, KGB-1, and SKN-1) and the *C. elegans* small RNA processing pathway. We know very little about what *clec-60* actually does, which is limited to the fact that *clec-60* appears to be secreted in high amounts into the *C. elegans* intestinal lumen in response to a number of Gram-positive bacterial infections, and likely plays a role in a protective host response.

To identify the components of the signaling pathways that activate *clec-60*, one could undertake a forward genetics approach to screen for constitutively active mutants using the *clec-60* transcriptional reporter. The goal of this experiment would be to identify a gain-of-function mutation in the most upstream receptor activated by an *Enterococcus* infection, which has led to the constitutive activation of this pathway. Loss-of-function mutations in negative regulators of this pathway will also be identified. The benefit of such a screen is that constitutively active mutants would be readily identifiable. Since these mutants are likely to be dominant mutations, one can easily screen F1 mutants for constitutive activation.

In fact, I did undertake such a screen, but I unfortunately encountered a number of technical difficulties. While performing the first part of this EMS mutagenesis for gain-of-function mutations that lead to the activation of the *clec-60* and *F35E12.5* reporters, I discovered that every F1 mutant isolated on the basis of constitutive activation of the *F35E12.5* reporter was sterile. This finding was reminiscent of a previous study in our lab that identified a trade-off between fertility and pathogen resistance, where the investigators performed a forward-genetics screen to identify *C. ele-*

gans mutants resistant to *P. aeruginosa* and also found that most of them were sterile [134]. In contrast to *F35E12.5*, I was able to isolate a number of mutants, however, in which *clec-60* was constitutively activated, and endogenous upregulation of *clec-60* was confirmed, demonstrating proof-of-principle of this method. However, this screen could not be pursued due to lack of time. As the only receptor definitively shown to be involved in immunity is DCAR-1 [246], which senses epidermal damage, this screen would certainly be worth pursuing.

As alluded to previously, *clec-60* is a gene downstream of many stress-related signaling pathways, including FSHR-1, an LRR-GPCR for which we know very little. The mutants isolated from the above described forward genetics screen may also be able to identify genes that are upstream and downstream of FSHR-1. Crossing the constitutively-activated *clec-60* mutants into various stress mutants, including mutants impaired in the potential stress and immune-related gene FSHR-1, would pinpoint where these mutants lie in respect to these various pathways.

As for FSHR-1, it will be extremely interesting to see whether *fshr-1* mutants – or even other immune signaling mutants, like *bar-1* – are hypersensitive to various cellular stresses, such as oxidative stress, heavy metal stress, ER stress, mitochondrial stress, or pore-forming toxins. This will be of interest because these mutants have been deemed "immune signaling mutants" because they are hypersusceptible to pathogenic infection and fail to upregulate infection-responsive genes; however, it is possible that the genes they regulate are actually related to a host stress response that is also mounted in response to the infection and required for defense. Further characterization of the FSHR-1 pathway through studies with the *fshr-1* mutant should also be informative. The genes downstream of *fshr-1* can be characterized via microarray or RNA-seq studies, at steady-state and in response to different pathogens. Furthermore, the ligand for FSHR-1 is unknown, and it is intriguing to think that this ligand – either of bacterial or host origin – is induced in response to pathogenic infection.

5.2 Potential caveats of the experimental system

One complication in understanding the influence of a particular bacterial species on *C. elegans* physiology is that bacteria not only act as a pathogen, but also comprise the diet of worms. This very fact makes some of the transcriptional profiling experiments, such as those described in this thesis, harder to interpret, as the rapid and robust activation of response genes in response to a pathogen can be interpreted as both an induction of putative immune effectors or, more trivially, a change in diet and metabolism.

A related issue worthy of mention is defining an appropriate *C. elegans* steady-state baseline, and what is an appropriate food source for transcriptional profiling studies. In this thesis, the control food source was heat-killed *E. coli* OP50 on brain-heart infusion (BHI) agar. I would have liked to use live *E. coli* on BHI as a control, but *C. elegans* fed on BHI-grown *E. coli* show a decrease in lifespan (a finding that suggests that *E. coli* may be pathogenic on rich media), and thus would not appropriately represent healthy *C. elegans* at steady-state [47]. *E. coli* grown in LB and then concentrated on nematode-growth media (NGM) would have also been improper as a baseline, as *Enterococcus* can only grow on rich media like BHI, and it would be inappropriate to have the baseline state reflect *C. elegans* grown on a different media from the other samples. For these reasons, I decided to use heat-killed *E. coli* on BHI as a baseline. However, one can imagine that *C. elegans* may be responsive to a dietary change from heat-killed *E. coli* to live *E. faecalis*, as the two food sources are vastly different (heat-killed vs. live, Gram-negative vs. Gram-positive, *etc.*) Thus, in future studies of the *C. elegans* pathogen response, where pathogenic bacteria also serve as food, it will be important to differentiate between dietary and pathogenic effects.

The issue of an appropriate baseline also arises for survival studies in *C. elegans* in determining whether a particular isolated mutant is defective in immunity or whether it generally has a decrease in lifespan. Traditionally, if a putative immune mutant showed a normal lifespan on *E. coli* OP50 (NGM) but was shown, compared to N2 wild-type worms, to have increased susceptibility to a par-

ticular pathogen, it was deemed to be specifically "immunocompromised." However, if the putative immune mutant showed a decreased survival on *E. coli* as well as the pathogen of interest, it was difficult to conclude whether the mutant was truly immunodeficient.

This poses some difficulties. First, it is now appreciated that not all *E. coli* are created equal, as different *E. coli* strains used as a "maintenance food source" for rearing *C. elegans* (namely the *E. coli* B strain OP50 and the K-12 strain HT115) have differences in carbohydrate content and fatty acid composition, and also differentially impact *C. elegans* metabolism[152,177]. As a result, there may be differential lifespans between N2 and putative immunodeficient mutants that are reared on different *E. coli* strains, as use of a particular *E. coli* strain may mask or unmask phenotypes linked to metabolism, fecundity, growth, or defense responses. Second, some immunodeficient mutants may exhibit a decrease in lifespan on *E. coli* because they are deficient at producing the necessary antimicrobial peptides and lysozymes to keep even *E. coli* a "non-pathogenic food source" in check in the intestine. Given that *E. coli* also has the potential to become pathogenic, exemplified by its ability to kill *C. elegans* when it is grown on rich media, the use of heat-killed *E. coli* to assess the lifespan of immunodeficient mutants is often used. This choice has its limitations too, as heat-killed *E. coli* are of a different texture and composition from live *E. coli*, and assessing lifespan on heat-killed *E. coli* may reveal differences in diet preference, behavior, and potentially metabolism associated with the putative immune mutant. Some researchers have also used chemically-defined axenic media to rear worms[147,183], though because most of *C. elegans* biology has been carried out using *E. coli* OP50 NGM plates, it continues to be the maintenance food and medium of choice. Nevertheless, it is important to keep in mind that different bacterial diets influence *C. elegans* development, reproduction, fat metabolism, and lifespan differentially, and there is no perfect baseline.

Most of the pathogenicity assays and genetic screens that have been used to study the defense response in *C. elegans* have primarily assessed mortality (*i.e.,* using killing assays), as it is much harder to assess morbidity. As a result, immune pathways that reduce morbidity will be overlooked in our studies. In the future, more assays to measure morbidity should be developed, and this can be achieved,

in part, through the generation of more host stress and immune response reporters, which will be very useful for genetic and chemical screens.

Finally, it is important to remember that most of the host-pathogen interactions studies in *C. elegans* have used human pathogens, and surprisingly there are many human pathogens that have been shown to infect *C. elegans*. These human pathogens are at best "accidental" pathogens from the *C. elegans* point of view. Further, in order to induce specific virulence traits or growth in human pathogens, they must be pre-grown at the optimal temperature of 37°C, a highly unphysiological temperature for *C. elegans*. This problem is not likely to arise using natural pathogens of *C. elegans*. However, it is only recently that natural pathogens have been described (*e.g.*, the Gram-negative bacterium *Microbacterium nematophilum*[66], the fungus *Drechmeria coniospora*[165], the microsporidian parasite *Nematocida parisii*[217], and most recently a nodavirus-like Orsay virus[42]. While we have gained many insights into *C. elegans* innate immunity and bacterial pathogenesis using human pathogens, where we have observed meaningful physiological interactions between the host and pathogen, one must consider that complementary studies using natural pathogens of wild nematodes, which have co-evolved with nematodes, will be able to offer further understanding into virulence mechanisms used to subvert host processes, or innate immune mechanisms used to thwart pathogen effector proteins.

These future studies will aid in the extension of our knowledge of innate immunity in *C. elegans* and lead to new areas of scientific inquiry. As *C. elegans* is a highly genetically-tractable organism that is additionally amenable to RNAi studies, one can appreciate the power of a dual genetic model to deconstruct host-pathogen interactions with great facility, and in greater detail than is possible in vertebrate models. It will be exciting to see how this new field of *C. elegans* innate immunity will evolve, perhaps to eventually characterize innate immune signaling pathways in mammals that previously had been ignored because they had been shrouded by the effects of other innate immune signaling pathways and a concomitant adaptive immune response. *C. elegans* infection models may continue to reveal surprising insights into the biology of infections that will further our understanding of the relationship between the mechanisms of infection and the host immune response and help

build an integrated view of microbial pathogenesis.

A

Materials and methods

A.1 Strains and growth conditions

All *C. elegans* strains were maintained on nematode growth media (NGM) and fed *E. coli* strain OP50, as previously described. The *C. elegans* strains used in this study can be found in Table A.1. Unless otherwise stated, the *E. coli*, *E. faecalis*, *E. faecium*, and *B. subtilis* strains used in this study correspond to strains OP50, MMH594, E007, and PY79 *sigF*::kan, respectively, and are listed in Table A.2.

Table A.1: *C. elegans* strains used in this study

Name	Genotype	Source
N2 (Bristol)	Wild-type	CGC
CF512	*fer-15*(b26)II; *fem-1*(hc17)IV	CGC
AU0285	*bar-1* (ga80)X, backcrossed 3×	CGC
AU0066	*pmk-1*(km25)IV, backcrossed 3×	K. Matsumoto
RB911	*fshr-1*(ok778)V, backcrossed 4×	Powell *et al.*[163]
AU131	*pmk-1*(km25)IV; *fshr-1*(ok778)V	Powell *et al.*[163]
	pmk-1(km25)IV;*bar-1*(ga180)X	J. Irazoqui
IG544	*nipi-3*(fr4)X	CGC
RB582	*mpk-2*(ok219)II	CGC

A.2 Nematode killing assays

For all enterococcal killing assays, starter cultures of *E. faecalis* or *E. faecium* strains were prepared by growth in BHI for 6-8 hours with shaking at 37°C. Afterward, 10 µL of log phase cultures were spread onto 35 mm brain-heart infusion (BHI) agar plates containing 10µg/ml kanamycin, and incubated at 37°C overnight (16-20 hours)[162]. Before use, the plates were allowed to equilibrate to room temperature. Approximately 40-50 late L4-staged *C. elegans* worms were then transferred from a lawn of *E. coli* OP50 on NGM to BHI medium-grown *Enterococcus*, taking care to transfer as little *ecoli* as possible from the maintenance plates to the killing plates. Nematodes were placed outside of the lawn on the bare agar. The plates were then incubated at 25°C, and every 24 hours, worms were examined for viability using a dissecting microscope. Worms that did not respond to a gentle touch with a platinum wire pick to the head, body, and tail were scored as dead. As *E. faecium*-infected *C. elegans* towards the end of their lives do not move much, even when prodded, special attention had to be paid to head movement and pharyngeal pumping to determine whether the nematodes were alive. Worms that did not move were scored as dead, counted, and picked off the killing plate.

Table A.2: List of bacterial strains used in this study

Strain	Species	Description	Source
OP50	*E. coli*	Ura$^-$ Str^R	G. Ruvkun
MMH594	*E. faecalis*	Clinical isolate, Esp$^+$	M. Gilmore
E007	*E. faecium*	Clinical isolate, Tet^R	M. Gilmore
FA2-2 pAM714	*E. faecalis*	Wild-type, with transposon Tn917	M. Gilmore
FA2-2 pAM771	*E. faecalis*	CylL-deficient non-hemolytic, with Tn917 insertion that blocks production of cytolysin toxin precursors	M. Gilmore
FA2-2 pAM9055	*E. faecalis*	Cyl-A deficient non-hemolytic, with Tn917 insertion in gene encoding activator protease	M. Gilmore
FA2-2 pAM710	*E. faecalis*	hyperhemolytic, due to dysregulating transposon Tn917 insertion	M. Gilmore
PY79	*B. subtilis*	*sigF*::kan	R. Losick
NCBI3610	*B. subtilis*	"wild" isolate, *sigF*::kan	R. Losick

Worms that were found to be still moving were scored as alive and were also counted.

For catalase treatment experiments, 1,000 U of catalase in 100 µL was spread onto solidified BHI agar. Each experimental condition was tested in triplicate. Kaplan-Meier log rank analysis was performed to determine the statistical significance of the difference in survival curves using OASIS[237], an online, publicly available tool that provides Kaplan-Meier estimates and mean/median survival time by based on censored survival data. P values < 0.05 were considered statistically significant.

A.3 RNAi feeding experiments

RNAi constructs were obtained from the publicly available Ahringer RNAi library[88]. All clones were verified by sequencing. For RNAi experiments, starter cultures of RNAi expressing HT115 bacterial clones were grown overnight in LB (carbenicillin and tetracycline) at 37°C, followed by further

growth for 4-6 hours in a larger volume of LB (carbenicillin) at 37°C. NGM plates containing 5 mM IPTG and 100 μg/mL carbenicillin were then seeded with the double-stranded RNAi-expressing HT115 bacteria[88]. The bacteria were induced over two days, after which synchronized L1 worms were added to the plates. Worms were fed through the L4 stage with dsRNA-expressing bacteria to target genes of interest. To obviate the issue of "bagging" confounding the pathogenicity assays, larvae were exposed to cdc-25.1 RNAi to sterilize worms prior to killing assays.

A.4 Defecation assays

N2 or *pmk-1* worms were grown to the L4 stage and were then picked to *Enterococcus* or *E. coli*-seeded plates and incubated at 25°C for 8 or 24 hours. For scoring, worms were then moved to room temperature, allowed to acclimate for 30-60 minutes, and their defecation phenotype was scored[213] by assessing the time between expulsions (which are preceded by posterior and anterior body wall muscle contraction, and the contraction of enteric muscles in a stereotyped, normally regular phenomenon). Defecation cycles were also followed in individual L4 worms for 30 consecutive cycles for some experiments. In general, more than 20 worms were scored and experiments were performed in duplicate. The significance of differences in values between conditions was determined using unpaired two-tailed Student t tests, with unequal variance.

A.5 RNA isolation

C. elegans N2 wild-type animals were synchronized by hypochlorite treatment and L1 arrested. Arrested L1 worms were allowed to grow on NGM media seeded with OP50 and grown at 20°C until they reached the young adult stage. Young adults were then washed three times in M9W buffer and transferred to BHI (10 μg/mL) plates seeded with heat-killed *E. coli* OP50, live *B. subtilis* PY79, live *E. faecalis* MMH594, or live *E. faecium* E007. Worms were treated as described for the killing assays, with the exception that approximately 2,000 worms were plated onto each 10-cm assay plate. After

8 hours at 25°C, the treated worms were washed three times in M9W, resuspended in TRI Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions, and frozen at -80°C. Once thawed, total RNA was purified using an RNeasy column (Qiagen). Three independent replicates of each treatment were isolated.

For the multiplexed gene expression profiling studies using NanoString nCounter, the same protocol was followed, except altering the *C. elegans* strain (genotype) or the type of bacteria, depending on the experiment. Between one and three independent replicates of each treatment were carried out.

For feeding of *C. elegans* with heat-killed bacteria, bacteria were pelleted by centrifugation, washed thrice in a large excess of M9W, concentrated 25x and incubated at 95°C for 90-120 minutes. The heat-killed bacterial suspension was equilibrated to room temperature before it was added to BHI agar plates and allowed to dry under a sterile hood with heat. Once dry, these plates were allowed to once again equilibrate to room temperature before worms were added to them.

A.6 NanoString nCounter analysis

RNA was analyzed by NanoString nCounter Gene Expression Analysis (NanoString Technologies) using a "CodeSet" designed in consultation with NanoString Technologies that contained probes for 72 *C. elegans* genes of interest. Probe hybridization, data acquisition and analysis were carried out according to instructions from NanoString. Each RNA sample was normalized to the housekeeping genes *snb-1*, *ama-1*, *act-1*, *pmp-3*, and *tba-1* using the nSolver Analysis software (NanoString Technologies).

A.7 Transmission electron microscopy

C. elegans were fixed overnight at 4°C in 2.5% glutaraldehyde, 1.0% paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 7.4 plus 3.0% sucrose. The cuticles were nicked with a razor blade

in a drop of fixative under a dissecting microscope to allow the fixative to penetrate. After 1 hour fixation at room temperature, the worms were fixed overnight at 4°C. After several rinses in 0.1 M cacodylate buffer, the samples were post-fixed in 1.0% osmium tetroxide in 0.1 M cacodylate buffer for one hr at room temp. They were rinsed in buffer and then in double distilled water and stained, *en bloc* in 2.0% aqueous uranyl acetate for 1 hour at room temperature. After rinsing in distilled water, the last rinse was carefully drawn off and the worms were embedded in 2.0% agarose in PBS for ease of handling.

The agarose blocks were dehydrated through a graded series of ethanol to 100%, then into a 1:1 mixture of ethanol:EPON overnight on a rocker. The following day, the agarose blocks were further infiltrated in 100% EPON for several hours and then were embedded in fresh EPON overnight at 60°C. Thin sections were cut on a Reichert Ultracut E ultramicrotome, collected on formvar-coated gold grids, post-stained with uranyl acetate and lead citrate, and viewed in a JEOL 1011 TEM at 80 kV equipped with an AMT digital imaging system (Advanced Microscopy Techniques, Danvers, MA). Transmission electron microscopy studies were carried out at the MGH Microscopy Core, Program in Membrane Biology.

A.8 Microarray analysis

RNA samples were prepared and hybridized to the Affymetrix *C. elegans* Genome Array GeneChips, following protocols and instructions previously described from Affymetrix, at the Joslin Advanced Genomics and Genetics Core (Boston, MA). Data were analyzed using GenePattern version 3.9.1 using RMA and quantile normalization. The expression profiles were compared using GenePattern to determine the fold change between conditions for each probe set and to generate a p-value using a modified t-test. Probe sets were considered differentially expressed if the fold change was 2-fold or greater and the p-value was less than 0.05. This approach also allowed for the generation of lists of genes that represented the gene signatures of the specific treatment conditions. Publicly available microarray datasets of *C. elegans* infection were compared by determining the overlap between the

different gene signatures, and the statistical significance of the gene set overlap was calculated using the hypergeometric test[243]. Area-proportional Venn diagrams were generated using the R package VennEuler.

A.9 Microscopy

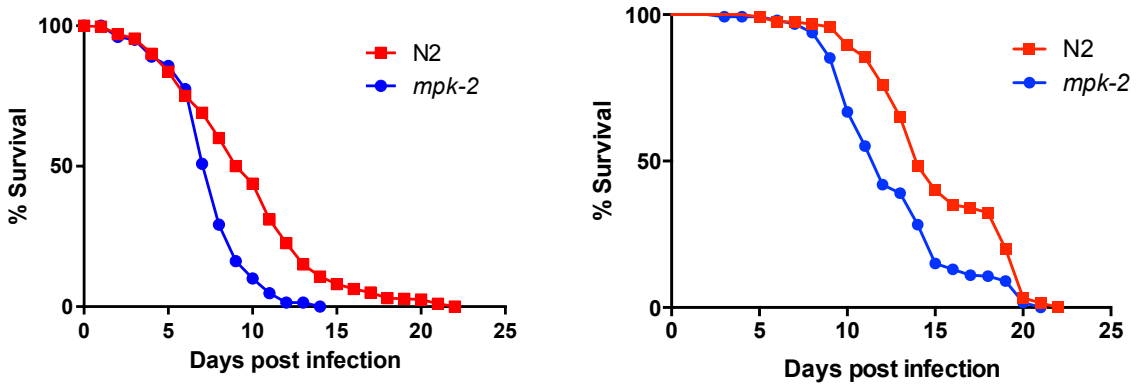
C. elegans were first mounted onto agar pads and paralyzed with 10 mM levamisole (Sigma). *C. elegans* were then observed by differential interference contrast imaging with Nomarski optics, imaged using a Zeiss AXIO Imager Z1 microscope, a Zeiss AxioCam HRm camera, and Axiovision 4.6 (Zeiss) software.

B

Related observations

B.1 The *C. elegans* MAP kinase MPK-2 is a putative immune signaling component

MPK-2 is a predicted serine/threonine kinase that is believed to act downstream of a receptor tyrosine kinase. While *mpk-2* RNAi knockdown yields no reported observable phenotype, it has been observed to be transcriptionally induced by a number of pathogens, including *M. nematophilum* and *S. aureus*. As a result, it has been speculated to function as a candidate regulatory component of the immune response in the intestine. From experiments that transcriptionally-profiled *C. elegans* infected with *E. faecalis* and *E. faecium*, I noticed that *mpk-2* is differentially induced by *E. faecalis* relative to *E. faecium* (Figure C.1).



(a) *E. faecalis* **(b)** *E. faecium*

Figure B.1: MPK-2 is required for defense against *E. faecium*. Effect of *E. faecium* infection on wild-type N2 and mutant *mpk-2 C. elegans* on *E. faecium* strain E007. *C. elegans* deficient in *mpk-2* exhibit a shortened lifespan upon infection with *E. faecalis* ($p = 4.40 \times 10^{-8}$) and *E. faecium* ($p = 1.80 \times 10^{-5}$).

To test whether *mpk-2* had a role in resistance to enterococcal infection, I infected wild-type N2 and *mpk-2* mutant worms with *E. faecalis* and *E. faecium*. I found that the *mpk-2* mutant shows enhanced susceptibility to infection with *E. faecalis* and *E. faecium* (Figure B.1). Future studies will be required to show whether the *mpk-2* is also hypersusceptible to infection with other pathogenic bacteria or fungi. Additionally, it will be important to determine whether the *mpk-2* mutant exhibits a normal lifespan on *E. coli*, and to assess whether it shows a defect in colonization resistance to bacterial infection. Epistasis analysis of *mpk-2* with previously characterized immune genes will be necessary to place the gene in a pathway. Furthermore, a transcriptional reporter will be required to ascertain the distribution of tissues in which *mpk-2* is expressed.

B.2 A heat-stable factor from *Bacillus subtilis* induces the *F35E12.5* reporter

The CUB-like gene *F35E12.5* was first studied by the Aballay lab at Duke University, after it was found to be positively regulated by PMK-1 and upregulated in response to *Y. pestis* infection. This gene was later shown to be involved in the transcriptional response to several bacterial pathogens.

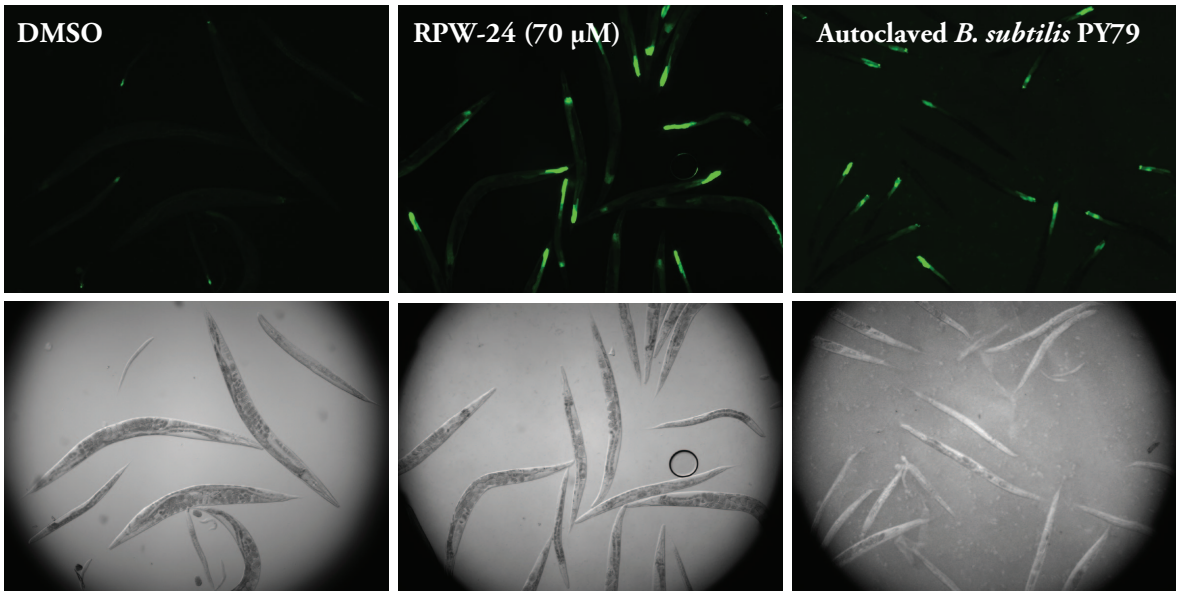


Figure B.2: Induction of the *F35E12.5* reporter by an insoluble extract from autoclaved *B. subtilis*. L4 *C. elegans* worms were placed on DMSO (vehicle control, left), RPW-24 (a small molecule known to induce the *F35E12.5* reporter, center), and autoclaved *B. subtilis* PY79 extract (right). Worms were imaged 20 hours post treatment.

Transgenic *C. elegans* animals carrying a transcriptional GFP reporter for the F35E12.5 gene were used to screen a number of compounds, which had previously been demonstrated to exhibit *P. aeruginosa* curing activity, for immunomodulatory activity. This method proved successful, as one compound from the set (called RPW-24) was shown to activate immunity via the PMK-1 p38 MAPK pathway[168].

I have observed that autoclaved *B. subtilis* extracts (Figure B.2), and to some degree, autoclaved *E. coli* extracts (not shown), greatly induced the p*F35E12.5*::GFP transcriptional reporter, with the greatest induction seen between 15 and 20 hours post infection. Neither live *B. subtilis* nor live *E. coli* elicited this response. Furthermore, *Enterococcus* extracts, from either *E. faecalis* or *E. faecium*, were not found to induce this reporter; autoclaving of *Enterococcus* extracts did not induce the reporter, either.

To determine in what fraction the *F35E12.5*-inducing activity was located, cultures of *B. subtilis* were centrifuged, washed thrice, autoclaved for 30 minutes, lysed by sonication (2 minutes), and

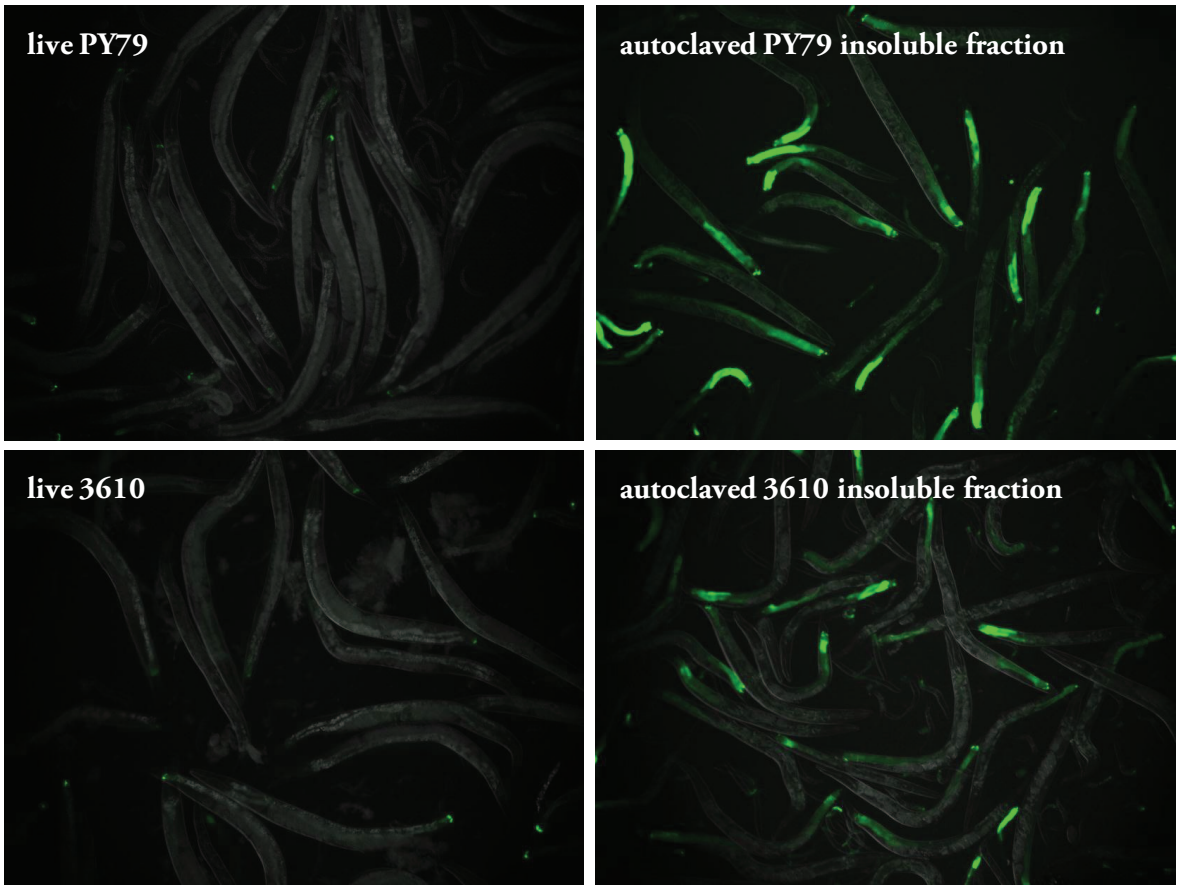


Figure B.3: A bacterial-derived factor from *B. subtilis* induces the *F35E12.5* immune reporter. L4 *C. elegans* worms were placed on two different strains of *B. subtilis*, PY79 (laboratory strain) and 3610 (wild isolate), which were either live (left panels) or heat-killed (autoclaved, insoluble extracts, right panels). Worms were imaged 20 hours post treatment.

then centrifuged at 30,000×g (15 minutes) to separate the soluble from insoluble fractions. These fractions were then tested on plates with *F35E12.5* reporter worms. Only the insoluble fraction of *B. subtilis* was found to induce *F35E12.5* (Figure B.3). These data suggest that a heat-stable motif or factor is released from lysed *B. subtilis* extracts, which is capable of inducing the *F35E12.5* reporter. However, further biochemical characterization will be required to identify the factor that induces the *F35E12.5* reporter.

C

Supplementary tables

Table C.1: *C. elegans* genes induced 2-fold or higher 8 hours after infection with *E. faecalis*, relative to *E. faecium* with $p < 0.05$

Affymetrix ID	Gene	Description	Fold change
192509_at	clec-60	C-type LECtin	19.61
184993_at	H02F09.3	hypothetical protein	18.52
183381_at	C50F7.5	hypothetical protein	11.63
184295_at	Y37H2A.14	hypothetical protein	9.9
184771_at	H02F09.2	hypothetical protein	9.62
190545_at	mpk-2	MAP Kinase	8.13
179858_at	C49C8.5	hypothetical protein	7.87
178202_at	F46B3.1	hypothetical protein	6.17
174347_s_at	C49C8.5	hypothetical protein	5.75
173015_s_at	clec-81	C-type LECtin	4.78

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Table C.1: (continued)

Probe ID	Gene	Description	Fold change
179498_at	fbxa-157	F-box A protein	4.72
185280_at	H43E16.1	hypothetical protein	4.42
189299_at		hypothetical protein, phospholipase	3.85
186179_at	K05F1.10	hypothetical protein	3.8
172184_x_at	clec-174	C-type LECtin	3.79
175317_s_at	E02D9.1	hypothetical protein	3.75
179373_at	C50F4.9	hypothetical protein	3.42
186289_at	F55G1.15	hypothetical protein	3.39
189132_at	E02D9.1	hypothetical protein	3.33
176044_at	Y41D4B.16	hypothetical protein	3.13
175212_at	clec-82	C-type LECtin	3.09
188358_at		hypothetical protein, amidase	3.07
192195_at	acs-2	fatty Acid CoA Synthetase family	3.01
177956_at	srd-53	Serpentine Receptor, class D (delta)	2.99
184777_s_at	K06A9.1	hypothetical protein	2.94
177375_at	M60.2	hypothetical protein	2.81
186886_s_at	Y18D10A.23	hypothetical protein	2.75
180453_at	F35A9.1	hypothetical protein	2.74
179499_s_at	fbxa-156	F-box A protein	2.7
172070_x_at	K06A9.1	hypothetical protein	2.7
183652_at	far-7	Fatty Acid/Retinol binding protein	2.69
183549_at	C49A9.5	hypothetical protein	2.54
175390_at	flp-16	FMRF-Like Peptide	2.51
180946_at	ilys-3	Invertebrate LYSozyme	2.51
184855_at	T23B12.8	hypothetical protein	2.51
172762_x_at			2.51
190307_s_at	his-10	HIStone	2.51
175278_at			2.5
190439_s_at	cav-2	CAVeolin	2.47
178918_at	T06D8.1	hypothetical protein, T06D8.1	2.46
184558_at	nlp-17	Neuropeptide-Like Protein	2.43
194259_x_at	K06A9.1	hypothetical protein	2.43
172731_x_at			2.43
173257_s_at			2.41
172766_x_at			2.4
178771_at	F59C6.14	hypothetical protein	2.4
179651_s_at	fbxa-156	F-box A protein	2.36
172748_x_at			2.34
172797_x_at			2.34
193419_at	F35B12.9	hypothetical protein	2.34
174957_s_at	oac-31	O-ACyltransferase homolog	2.33
189100_at	clec-187	C-type LECtin	2.32
172136_x_at			2.27
172767_x_at			2.27
193098_at	cin-3.3	human CLN (neuronal ceroid lipofuscinosis) related	2.27
194056_s_at	clec-62	C-type LECtin	2.27
186124_at	T02B11.4	hypothetical protein	2.25

Continued on next page

Table C.1: (continued)

Probe ID	Gene	Description	Fold change
188496_s_at		hypothetical protein, permease	2.25
172309_x_at	C08E3.1	hypothetical protein	2.25
172747_x_at			2.25
180145_s_at	Y51H4A.7	hypothetical protein	2.23
172970_x_at			2.22
176939_at	spp-23	SaPosin-like Protein family	2.22
177100_at	clec-71	C-type LECtin	2.22
189150_at	kgb-2	Kinase, GLH-Binding	2.22
172214_x_at	flp-15	FMRF-Like Peptide	2.21
172913_x_at			2.21
180342_at	F35E12.6	hypothetical protein	2.2
188123_at		hypothetical protein, lipase	2.2
177368_at	M60.2	hypothetical protein	2.19
187767_at	otpl-3	OToPetrin-Like	2.17
188975_at	C15A11.7	hypothetical protein	2.17
176069_s_at	cls-1	hypothetical protein	2.16
173558_at	ZC443.3	hypothetical protein	2.15
187729_s_at	tre-5	TREhalase	2.15
188068_at	nlp-10	Neuropeptide-Like Protein	2.14
172352_at	C33A12.4	hypothetical protein	2.13
193200_s_at	gale-1	UDP-GALactose 4-Epimerase	2.12
184397_s_at	C46H11.2	hypothetical protein	2.11
176030_at	C06E1.7	hypothetical protein	2.11
172765_x_at			2.1
174748_at	T06D8.1	hypothetical protein	2.1
171849_x_at	flp-15	FMRF-Like Peptide	2.09
185413_at	Y75B12B.3	hypothetical protein	2.09
178432_s_at	ZC443.3	hypothetical protein	2.09
172335_x_at			2.05
178872_at	C25F9.12	hypothetical protein	2.05
180128_at	Y51H4A.8	hypothetical protein	2.05
172627_x_at	Y105C5A.12	hypothetical protein	2.04
173008_s_at	nhr-88	Nuclear Hormone Receptor family	2.04
174684_s_at	H43E16.1	A. faecalis PHB depolymerase; zinc finger protein 40, H43E16.1	2.03
193741_at	zig-6	2 (Zwei) IG-domain protein	2.02
173224_s_at	ins-11	INSulin related	2.02
179022_at	C50F4.1	hypothetical protein	2.02

Table C.2: *C. elegans* genes induced 2-fold or higher 8 hours after *E. faecium* infection, relative to *E. faecalis*, with $p < 0.05$

Affymetrix ID	Description	Public name	Fold Change
177544_at	hypothetical protein	F58E6.7	0.26
185275_at	CaDmium Responsive	cdr-5	0.32
185270_at	hypothetical protein	irg-3	0.39
188487_s_at	MeTaLlothionein	mtl-2	0.41
172744_at	MeTaLlothionein	mtl-1	0.43
186349_at	hypothetical protein	Y51H7C.1	0.45
181972_at	GRounDhog (hedgehog-like family)	grd-3	0.46
183850_at	hypothetical protein	Y48E18.8	0.46
173783_at	hypothetical protein, aminopeptidase		0.46
189151_s_at	hypothetical protein, aminopeptidase		0.48

Table C.3: Genes upregulated by both *E. faecalis* and *S. aureus* infection, induced 2-fold or higher, with $p < 0.05$

Affymetrix ID	Description	Public name	Fold Change (*E. faecalis* vs. HK *E. coli*)	Fold Change (*S. aureus* vs. HK *E. coli*)
191759_at	Flavin-containing MonoOxygenase	fmo-2	81.11	73.67
192195_at	fatty Acid CoA Synthetase family	acs-2	36.07	3.01
186971_at	hypothetical protein	C23G10.11	13.54	4.75
189299_at	hypothetical protein		11.04	13.77
190978_at	SOrbitol DeHydrogenase family	sodh-1	7.58	9.24
183652_at	Fatty Acid/Retinol binding protein	far-7	7.54	5.46
184624_s_at	hypothetical protein	C25H3.10	6.86	3.57
182470_at	hypothetical protein	F09F7.6	5.17	3.46
189221_at	CYtochrome P450 family	cyp-37B1	4.94	5.69
193914_at	Gamma Butyrobetaine Hydroxylase	gbh-2	3.54	2.76
180946_at	Invertebrate LYSozyme	ilys-3	3.4	15.14
190009_at	hypothetical protein, ERG-3 like protein	F49E12.10	3.15	2.48
188078_at	hypothetical protein	lipl-2	2.94	3.66
182444_at	hypothetical protein	F18G5.6	2.89	2.17
178432_s_at	hypothetical protein	ZC443.3	2.86	2.48
173480_s_at	CYtochrome P450 family	cyp-32B1	2.67	3.42
173691_s_at	hypothetical protein	ZK688.2	2.63	2.43
180721_at	Invertebrate LYSozyme	ilys-2	2.53	7.41

Table C.4: Genes upregulated by both *E. faecalis* and *P. aeruginosa* infection, induced 2-fold or higher, with $p < 0.05$

Affymetrix ID	Description	Public name	Fold Change (*E. faecalis* vs. HK *E. coli*)	Fold Change (*P. aeruginosa* vs. *E. coli*, 8 hrs)
192195_at	fatty Acid CoA Synthetase family	acs-2	36.07	3.26
184993_at	hypothetical protein	H02F09.3	8.99	6.55
190545_at	MAP Kinase	mpk-2	8.74	2.06
183652_at	Fatty Acid/Retinol binding protein	far-7	7.54	2.85
185270_at	hypothetical protein	irg-3	4.81	11.43
186179_at	hypothetical protein	K05F1.10	3.39	2.07
179457_at	hypothetical protein	B0024.4	3.09	4.91
178441_at	hypothetical protein	C34C6.7	2.45	2.54
190307_s_at	HIStone	his-10	2.24	2.78
176135_s_at	F-box A protein	fbxa-91	2.02	2.3

Table C.5: Genes upregulated by both *E. faecalis* and *C. albicans* infection, induced 2-fold or higher, with $p < 0.05$

Affymetrix ID	Description	Public name	Fold Change (*E. faecalis* vs. HK *E. coli*)	Fold Change (*C. albicans* vs. HK *E. coli*)
188496_s_at	hypothetical protein		12.91	2.18
183652_at	Fatty Acid/Retinol binding protein	far-7	7.542	2.34
184624_s_at	hypothetical protein	C25H3.10	6.862	3.62
189221_at	CYtochrome P450 family	cyp-37B1	4.944	2.34
179094_s_at	hypothetical protein	T16G1.4	3.822	2.85
173558_at	hypothetical protein	ZC443.3	3.712	2.4
173008_s_at	Nuclear Hormone Receptor family	nhr-88	3.584	2.13
180946_at	Invertebrate LYSozyme	ilys-3	3.395	2.4
190359_at	hypothetical protein	acs-7	2.884	2.66
178432_s_at	hypothetical protein	ZC443.3	2.861	2.49
192737_at	SCP-Like extracellular protein	scl-2	2.756	2.95
185969_s_at	hypothetical protein	C32F10.4	2.663	2.06
192181_at	hypothetical protein, vacuolar processing enzyme like	T28H10.3	2.62	2.17
178441_at	hypothetical protein	C34C6.7	2.454	2.02
178918_at	hypothetical protein	T06D8.1	2.19	2.88
190999_at	hypothetical protein	T20B3.1	2.151	2.02

Table C.6: Genes upregulated by both *E. faecalis* and *C. albicans*, induced 2-fold or higher, with $p < 0.05$

Affymetrix ID	Description	Public name	Fold Change (*E. faecalis* vs. *E. faecium*)	Fold Change (*M.nematophilum* vs. avirulent)
192509_at	C-type LECtin	clec-60	19.61	6.21
184993_at	hypothetical protein	H02F09.3	18.52	5.03
183381_at	hypothetical protein	C50F7.5	11.63	27.03
184295_at	hypothetical protein	Y37H2A.14	9.9	3.17
190545_at	MAP Kinase	mpk-2	8.13	3.08
179858_at	hypothetical protein	C49C8.5	7.87	2.57
174347_s_at	hypothetical protein	C49C8.5	5.75	2.4
186179_at	hypothetical protein	K05F1.10	3.8	2.16
172184_x_at	C-type LECtin	clec-174	3.79	3.13
175317_s_at	hypothetical protein	E02D9.1	3.75	3.47
179373_at	hypothetical protein	C50F4.9	3.42	5.49
189132_at	hypothetical protein	E02D9.1	3.33	2.01
175212_at	C-type LECtin	clec-82	3.09	2.39
192195_at	fatty Acid CoA Synthetase	acs-2	3.01	2.72
183652_at	Fatty Acid/Retinol binding protein	far-7	2.69	2.8
173257_s_at			2.41	2.6
174957_s_at	O-ACyltransferase homolog	oac-31	2.33	3.22
194056_s_at	C-type LECtin	clec-62	2.27	2.12
188975_at	hypothetical protein	C15A11.7	2.17	2.73
173224_s_at	INSulin related	ins-11	2.02	3.53

Table C.7: Enrichment of gene families among genes differentially regulated by both *E. faecalis* and *E. faecium* infection relative to heat-killed *E. coli*, based on GO-terms.

UPREGULATED

Term	Description	Count	% enrichment	*p*-value
GO:0009055	electron carrier activity	11	9.40	3.80×10^{-6}
GO:0055114	oxidation reduction	12	10.26	4.59×10^{-5}
GO:0005506	iron ion binding	9	7.69	9.56×10^{-4}
GO:0004499	flavin-containing monooxygenase activity	3	2.56	1.26×10^{-3}
GO:0050662	coenzyme binding	7	5.98	1.65×10^{-3}
GO:0050660	FAD binding	5	4.27	1.76×10^{-3}
GO:0050661	NADP or NADPH binding	3	2.56	4.55×10^{-3}
GO:0048037	cofactor binding	7	5.98	8.50×10^{-3}
GO:0031300	intrinsic to organelle membrane	3	2.56	9.44×10^{-3}

DOWNREGULATED

Term	Description	Count	% enrichment	*p*-value
GO:0005529	sugar binding	5	5.05	4.71×10^{-3}
GO:0030246	carbohydrate binding	6	6.06	6.37×10^{-3}

Table C.8: Enrichment of protein domains among genes differentially regulated by both *E. faecalis* and *E. faecium* infection relative to heat-killed *E. coli*, based on GO-terms for InterPro protein domains.

UPREGULATED

Term	Description	Count	% enrichment	*p*-value
IPR000960	Flavin-containing monooxygenase FMO	3	2.56	9.25×10^{-4}
IPR017972	Cytochrome P450, conserved site	5	4.27	1.64×10^{-3}

DOWNREGULATED

Term	Description	Count	% enrichment	*p*-value
IPR001304	C-type lectin	7	7.07	3.54×10^{-3}
IPR016186	C-type lectin-like	6	6.06	9.10×10^{-3}

Table C.9: Enrichment of gene families among genes differentially regulated by *E. faecalis* infection but not *E. faecium* relative to heat-killed *E. coli*, based on GO-terms.

UPREGULATED

Term	Description	Count	% enrichment	p-value
GO:0015837	amine transport	5	3.45	4.21×10^{-5}
GO:0015849	organic acid transport	4	2.76	9.97×10^{-4}
GO:0030554	adenyl nucleotide binding	17	11.72	1.91×10^{-2}
GO:0006811	ion transport	9	6.21	2.07×10^{-2}
GO:0003779	actin binding	4	2.76	2.30×10^{-2}
GO:0006812	cation transport	7	4.83	2.62×10^{-2}
GO:0030421	defecation	3	2.07	3.14×10^{-2}

DOWNREGULATED

Term	Description	Count	% enrichment	p-value
GO:0003779	actin binding	3	5.17	1.47×10^{-2}
GO:0008092	cytoskeletal protein binding	3	5.17	2.22×10^{-2}
GO:0040018	positive regulation of multicellular organism growth	5	8.62	1.79×10^{-2}

Table C.10: Enrichment of protein domains among genes differentially regulated by *E. faecalis* infection but not *E. faecium* relative to heat-killed *E. coli*, based on GO-terms for InterPro protein domains.

UPREGULATED

Term	Description	Count	% enrichment	p-value
IPR004841	Amino acid permease-associated region	3	2.07	9.39×10^{-3}
IPR002900	Protein of unknown function DUF38, Caenorhabditis species	7	4.83	8.55×10^{-3}
IPR001628	Zinc finger, nuclear hormone receptor-type	7	4.83	1.73×10^{-2}
IPR008271	Serine/threonine protein kinase, active site	6	4.14	2.48×10^{-2}

DOWNREGULATED

Term	Description	Count	% enrichment	p-value
IPR002213	UDP-glucuronosyl/UDP-glucosyltransferase	4	6.90	2.03×10^{-3}
IPR007284	Ground-like region	3	5.17	7.72×10^{-3}

Table C.11: Enrichment of gene families among genes differentially regulated by *E. faecium* infection but not *E. faecalis* relative to heat-killed *E. coli*, based on GO-terms.

UPREGULATED

Term	Description	Count	% enrichment	p-value
GO:0006259	DNA metabolic process	21	9.05	2.32×10^{-11}
GO:0008026	ATP-dependent helicase activity	14	6.03	5.86×10^{-11}
GO:0005694	chromosome	11	4.74	3.69×10^{-9}
GO:0006974	response to DNA damage stimulus	13	5.60	5.08×10^{-8}
GO:0003006	reproductive developmental process	39	16.81	9.57×10^{-7}
GO:0033554	cellular response to stress	13	5.60	1.51×10^{-6}
GO:0005524	ATP binding	36	15.52	2.17×10^{-6}
GO:0032559	adenyl ribonucleotide binding	36	15.52	2.28×10^{-6}
GO:0040010	positive regulation of growth rate	60	25.86	8.53×10^{-6}
GO:0007548	sex differentiation	35	15.09	9.17×10^{-6}
GO:0030554	adenyl nucleotide binding	36	15.52	1.05×10^{-5}
GO:0043232	intracellular non-membrane-bounded organelle	15	6.47	1.16×10^{-5}
GO:0043228	non-membrane-bounded organelle	15	6.47	1.16×10^{-5}
GO:0007126	meiosis	14	6.03	1.76×10^{-5}
GO:0040035	hermaphrodite genitalia development	31	13.36	4.50×10^{-5}
GO:0002009	morphogenesis of an epithelium	19	8.19	1.22×10^{-4}
GO:0034061	DNA polymerase activity	5	2.16	1.91×10^{-4}
GO:0048729	tissue morphogenesis	19	8.19	2.05×10^{-4}
GO:0009791	post-embryonic development	56	24.14	3.74×10^{-4}
GO:0002164	larval development	54	23.28	9.90×10^{-4}

DOWNREGULATED

Term	Description	Count	% enrichment	p-value
GO:0031974	membrane-enclosed lumen	4	6.06	4.26×10^{-3}
GO:0044432	endoplasmic reticulum part	3	4.55	6.29×10^{-3}
GO:0050662	coenzyme binding	5	7.58	7.04×10^{-3}

Table C.12: Enrichment of protein domains among genes differentially regulated by *E. faecium* infection but not *E. faecalis* relative to heat-killed *E. coli*, based on GO-terms for InterPro protein domains.

UPREGULATED

Term	Description	Count	% enrichment	*p*-value
IPR014021	Helicase, superfamily 1 and 2, ATP-binding	12	5.17	1.16×10^{-9}
IPR000467	D111/G-patch	5	2.16	2.43×10^{-5}
IPR001878	Zinc finger, CCHC-type	5	2.16	1.15×10^{-3}
IPR017972	Cytochrome P450, conserved site	6	2.59	2.82×10^{-3}

DOWNREGULATED

Term	Description	Count	% enrichment	*p*-value
IPR001304	C-type lectin	6	9.09	2.84×10^{-3}

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